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PHARMACOLOGICAL AND BIOCHEMICAL ASPECTS OF BILE ACID SYNTHESIS IN THE ISOLATED PERFUSED RAT LIVER

by

Craig Leigh Bentzen

A Dissertation Submitted to the Faculty of the

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GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my direction by Craig Leigh Bentzen

entitled Pharmacological and Biochemical Aspects of Bile Acid Synthesis in the Isolated Perfused Rat Liver

be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

K. Cans Bruder — March 17. 76

Dissertation Director — Date

After inspection of the final copy of the dissertation, the following members of the Final Examination Committee concur in its approval and recommend its acceptance:*

March 17, 1976

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*This approval and acceptance is contingent on the candidate's adequate performance and defense of this dissertation at the final oral examination. The inclusion of this sheet bound into the library copy of the dissertation is evidence of satisfactory performance at the final examination.

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SIGNED: Craig L. Bentzen

To Sharon

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ABSTRACT

In the isolated perfused rat liver substrates of different oxidative potential (10 mM pyruvate, 10 mM dextrose, 10 mM lactate) were found to influence the rate of bile flow. Pyruvate and dextrose perfused livers had significantly higher bile flow rates compared to lactate perfused livers. These differences in bile flow rates produced were found to be due to the particular selective ability of the substrates to affect biliary sodium secretion. Although there was no observed effect of these substrates upon total bile acid synthesis and secretion, the individual bile acids synthesized and secreted by these perfused livers were dependent upon the particular substrate utilized during perfusion. This finding indicates that substrates or the redox state produced by the substrates can influence the individual pathways from cholesterol to the respective bile acids. When comparing the ratio of cholic acid to chenodeoxycholic acid plus metabolites, as affected by pyruvate, dextrose, and lactate, pyruvate produced the highest ratio, 0.845±0.05, demonstrating that pyruvate as a substrate was the most effective in directing synthesis toward the cholic acid pathway. Dextrose perfused livers had a lower mean ratio of 0.635±0.034 and lactate the lowest mean ratio of 0.310±0.06. The basic alterations in individual bile acid profiles in favor of cholic acid synthesis, as observed in response to a high oxidative state and when considering the biochemical and clinical findings, could be capable of inducing a

series of events leading to potential biliary lithogenisity. In order to study the effects of substrates upon the overall process of cholesterol turnover in a more direct and more physiological fashion, including *in vivo* experimentation, a [24,25-^{3}H]-cholesterol was synthesized from unlabeled demosterol. Upon [24,25-^{3}H]-cholesterol oxidation the respective hydroxylases and dehydrogenases release tritium from the compound into ^3H_2O. Since the tritiated water produced is freely exchangeable with cellular and extracellular water, the rate of tritiated water appearance in the perfusion medium of the isolated liver or the incubation medium of isolated suspended hepatocytes is identical to the rate of cholesterol sidechain oxidation and cholesterol turnover rates in the respective liver preparations. In intact animals the rate of appearance of ^3H_2O in the urine also represents the rate of cholesterol turnover to bile acids in the *in vivo* state. It was possible to segregate the actual process of cholesterol oxidation to bile acids from other processes such as conjugation and secretion using the [24,25-^{3}H]-cholesterol. This labeled cholesterol facilitated the study of the direct effects of these substrates upon cholesterol turnover to bile acids in the isolated liver and isolated suspended hepatocytes. These studies indicated the pyruvate (10 mM) as compared to lactate (10 mM) produced a higher rate (19.9-25.6%) of cholesterol turnover, although this was not detected by an increased biliary secretion of bile acids as determined by the 3α-steroid dehydrogenase reaction. This data then suggests that there is a rate limiting process in these preparations which is beyond the actual cholesterol sidechain oxidation. In

addition, the use of this labeled cholesterol in isolated liver cells and in intact animals under different thyroid states demonstrated the potential of this compound in studying the effects of other endogenous and exogenous compounds upon cholesterol turnover to bile acids as measured by the rate of cholesterol sidechain oxidation.

CHAPTER 1

INTRODUCTION

The gross structure of the liver, its tissue organization, and the cytologic and cytochemical features of the several cell types reflect its diverse and highly specialized functions (Elias and Sherrick, 1969). The liver is both a ducted gland, which secretes bile into the intestinal lumen and a synthetic tissue, which produces many blood proteins and lipids. In addition, the organ monitors and controls, by storage or interconversion, the plasma concentrations of a multitude of small molecules involved in metabolism. Of equal importance, the liver is the center for alteration of certain hormones, drugs, and sequesters materials of various origins and dimensions from the blood stream. The secretory, metabolic, and phagocytic operations are largely independent under normal conditions and are resilient to many changes including relatively severe pathological affront. No other organ can regenerate damaged tissue or so dramatically rearrange functions to meet demands placed upon it (Becker, 1970). This functional versatility and resiliency describes the liver as a remarkable organ and merits study into the mechanism of these complex processes.

The importance of bile acids for normal fat absorption in the gut has been recognized for years (Verzar and McDougal, 1936). Over one hundred years ago it was noted that bile in the presence of pancreatic lipase was important for normal fat absorption and that the

products of lipolysis stimulated the rate of fat absorption (Bidder and Schmidt, 1852). The detergent qualities of bile salts with their hydrophilic and lipophilic portions lead, under the appropriate conditions, to a physical state which has been termed "micellar solution" having the capability of solubilizing certain lipids (Hofmann and Borgstrom, 1962). In order to form such a micellar solution, bile salts must be present in an aqueous phase at a certain concentration referred to as the critical micellar concentration. The formation of a micellar solution is also dependent upon a critical temperature and a critical pH range (Small, 1968). The behavior of bile salt micelles is quite different from micelles formed by other detergents. Bile salt micelles are smaller, more highly charged and of different stucture (Ekwall, Sten, and Norman, 1956), and also contain a variety of other soluble and insoluble lipid substances. These mixed micelles incorporate appreciable amounts of important insoluble molecules such as fat soluble vitamins, cholesterol, phospholipids and monoglycerides, thus solubilizing them in the fluids of the bile and intestine (Holt, 1972). The bile acids have thus been regarded as possessing a dual function: 1) acting to solubilize dietary lipids in the intestine and thus promoting their absorption; 2) acting to solubilize lecithin and cholesterol in bile and thus aiding in the excretion of these otherwise insoluble endogenous lipids (Hofmann and Small, 1967).

That bile acids are themselves metabolites or end-products of cholesterol metabolism was not established until 1943 with the demonstration by Bloch, Berg, and Rittenberg (1943) that isotopically

labeled cholesterol was converted to cholic acid. The subsequent studies of Siperstein and Jayko (1952) and Bergstrom (1952) established that this catabolic pathway represented a major mechanism for the degradation and excretion of cholesterol. Thus, a rather interesting relationship exists between the bile acids and cholesterol in that the cholesterol metabolites, bile acids, are essential in maintaining both absorption and excretion of the parent compound, cholesterol.

Since bile acids themselves are absorbed from the small intestine, they are carried in the portal blood back to the liver where they are resequestered and resecreted into the bile. This enterohepatic circulation is so efficient in man that a relatively small pool of bile acids (between 2.5 and 4.0 grams) is cycled through the liver and intestine from 6-10 times a day, yet only a small amount of approximately 0.5 grams is lost in the feces during the same time (Small, Dowling, and Redinger, 1972). Under normal conditions bile acid homeostasis is maintained by the liver synthesizing an equivalent amount of bile acids to that lost in the feces.

Hepatic bile acid secretion is a fundamental step in the physiology of bile formation itself and has a profound influence on the volume and composition of bile. Normal hepatic bile consists of approximately 95% water and 5% solids. Of the solid components, bile salts, lecithin, and cholesterol comprise the major organic, and sodium salts the major inorganic components (Wheeler, 1968). Active bile acid secretion provides in part osmotic drive for cannicular water output and promotes biliary excretion of other solutes by solvent drag and

diffusion (Wheeler, Ross, and Bradley, 1968). There is also evidence for a bile acid-independent mechanism of cannicular water output which is most likely driven by active sodium transport (Boyer, 1971). Bile acid secretion is also the major driving force for biliary lecithin excretion (Shersten et al., 1971).

Interest in the toxicity of bile is not new. Bile has been equated with "poison" and as pointed out by Horrall (1938), physicians have been concerned with its toxicity since the days of Hippocrates and Galen. It is now recognized that the toxicity of bile is primarily related to the bile acid fraction. In addition, it has become apparent that several of the individual free bile acids, when found in excess, are associated with abnormal function and structure of organs, cells, and subcellular constituents (Holsti, 1962; Dietschy, 1967).

In the last decade the medical and scientific journals have contained many papers on the role of bile acids in disease. These studies have established that disturbed bile acid metabolism is responsible for prominent symptoms of several diseases, many of which are quite common. The following are a number of disease states in which bile salts have been implicated: some disorders of the terminal ileum, the stagnant or blind loop syndrome, cirrhosis of the liver, acute hemorrhagic pancreatitis, gastritis and gastric ulcers, tropical sprue, idiopathic steatorrhea, nonspecific diarrhea, disseminated sclerosis, cholestasis, hypercholesterolemia or cholelithiasis (Palmer, 1972).

The implications of the work discussed are associated with cholelithiasis and thus discussion of diseases in which bile salts

play an important role will be limited in this dissertation to the pathogenesis of cholelithiasis or gallstone formation.

In most cases gallstone formation is associated with cholesterol insolubility in bile fluid since cholesterol is the primary constituent of these stones (Sutor and Wooley, 1971). Furthermore, bile obtained from patients with cholesterol stones is usually saturated with cholesterol (Biss, Kang-Jey, and Mikkelson, 1971; Dam, Kruse, and Kallenhauge, 1967; Sarles, Hauton, and Planche, 1970). The presence of cholesterol in crystalline form in bile shows that at some time in the past the capacity of bile to dissolve cholesterol was exceeded. As was stated previously, bile salts are important for normal biliary solubilization of cholesterol, so that disorders of bile acid metabolism may result in abnormal lithogenic bile or a bile containing too much cholesterol relative to its content of bile salts and lecithin. Production of such bile is presumably the first stage in gallstone formation, the second being nucleatinon and precipitation of cholesterol crystals, and the third being growth and agglomeration in the gallbladder (Small, 1970).

Cholesterol excess cannot be defined in terms of an absolute concentration of cholesterol but only in terms of the relative proportions of cholesterol, bile salts and lecithin. It cannot even be defined accurately by the ratio of cholesterol to bile acid plus lecithin since the last two components having differing actions, are not additive (Holzbach, Marsh, and Holan, 1971). It is easiest to express the composition of a three component mixture using a triangular coordinate

phase diagram (Figure 1). In this phase diagram the physical state of cholesterol in bile is determined only by the proportions of cholesterol, lecithin and bile salts, provided the water content is between 75-96% (Redinger and Small, 1972). In 1968 Admirand and Small (1968) applied this techique to hepatic bile samples obtained at laparotomy from patients with cholesterol stones or mixed gallstones. Their results showed a clear separation between control and gallstone patients. The control subjects had biles with a compostition below the ABC line of maximum cholesterol solubility. In contrast, the biles from gallstone patients without microcrystals fall on or very near this line and the biles from gallstone patients with microcrystals fell well above the ABC line. This was the first study that demonstrated a consistent difference in bile composition between gallstone patients and controls.

The usefulness of such an index for prescreening potential gallstone patients was substantiated by the analysis of, at that time, a stone free population of young South West American Indian women who had a 70% expectancy of developing gallstones in the future. This analysis showed that half the subjects of this group already had bile that was lithogenic and thus were predisposed to gallstone formation (Sarpliner et al., 1970).

In 1970 two groups of workers confirmed the concept that the bile of gallstone patients is lithogenic before it enters the gallbladder. Using the triangular coordinates, Vlahcevic, Bell, and Swell (1970) found the composition of hepatic bile to be essentially the same

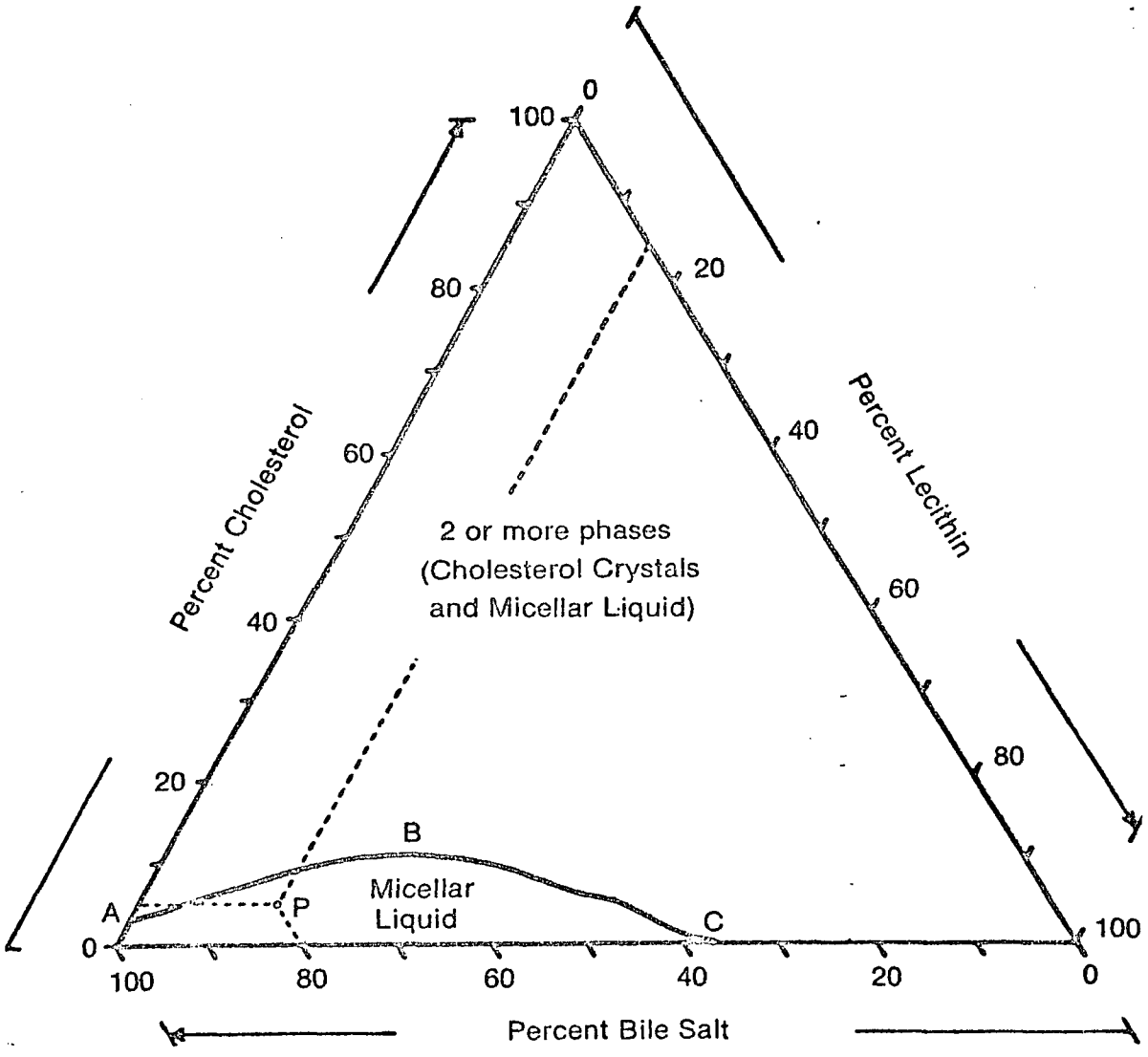

Figure 1. Representation of the three major components of bile (bile salts, cholesterol, and lecithin) as a triangular coordinate phase diagram.

Each component is expressed as percentage mole of total bile salt, cholesterol and lecithin. Line ABC represents maximum solubility of the cholesterol in varying mixtures of bile salts and lecithin. Point P represents bile composition containing 5% cholesterol, 15% lecithin, and 80% bile salt and falls within the zone of a single phase of micellar liquid. Bile having a composition falling above the ABC line would contain excess cholesterol in either super-saturated or precipitated form.

as that of gallbladder bile from the same gallstone patient. Small and Rapo (1970), however, found that in cases where hepatic bile was clearly over the line of cholesterol solubility, gallbladder bile was right on the limiting line. Despite this disagreement, these studies undoubtedly end the long argument as to the role of the gallbladder in the production of lithogenic bile. It can now be stated that the gallbladder is merely the innocent victim of a delinquent liver and only the site where, for reasons of flow characteristics, crystal nucleation and growth can take place.

The basis of gallstone disease is therefore the secretion by the liver of bile containing an excess of cholesterol in relation to bile salts and lecithin. Such an abnormality could arise from the secretion of an excess of cholesterol or from the secretion of inadequate amounts of bile salts and/or lecithin to balance a normal load of cholesterol.

Analysis of tube-aspirated duodenal contents suggest that most gallstone patients do not secrete excessive quantities of cholesterol but do secrete subnormal quantities of lecithin (Swell, Bell, and Vlahcevic, 1971). Since lecithin secretion is largely dependent on bile salt secretion, as previously discussed, this implied that deficient secretion of bile salts is the major underlying cause.

Since the vast majority of secreted bile salts are recirculated rather than newly synthesized, the main determinant of bile salt secretion has been considered to be the size of the circulating pool (Percy-Robb and Boyd, 1968). It has been shown that the total bile

salt pool is reduced to an average of 1.3 g or little more than half of normal in male patients with gallstones present in radiologically functional gallbladders. Heaton and Read (1969) also found a similar reduction in female patients with gallstones. Reduced pool sizes have also been reported in young American Indian women with a high probability of developing cholelithiasis but having normal gallbladders (Bell et al., 1972). These investigators demonstrated that subjects who secrete supersaturated bile in respect to cholesterol have a significantly reduced bile salt pool. Thus it seems very likely that the basis of lithogenic bile, at least in part, is due to a reduced bile salt pool size.

Over the last few years several investigators (Vander Linden, 1971; Heller and Boucher, 1973) have indicated that the bile of gallstone patients compared with control patients has a lower trihydroxy to dihydroxy bile acid ratio, although this was not confirmed by Thistle and Schoenfield (1971). This change in ratio was attributed to an increased proportion of deoxycholate. Even though it had been speculated that relative excess of dihydroxy bile salts may be lithogenic, on the grounds that the dihydroxy micelle is less polar and therefore less stable than the trihydroxy micelle (Burnett, 1965), it was experimentally found that dihydroxy bile salts, and especially deoxycholate, have a superior capacity for solubilizing cholesterol (Earnest and Admirand, 1971). In any case, the etiology of the lower trihydroxy/dihydroxy ratio seen in bile of gallstone patients is not well understood.

One explanation, which may be oversimplified, could be based on the mere difference of two pools of identical composition but of different size. After a given period of time assuming that the capacity of the gut flora for conversion of cholic acid to deoxycholic acid (a secondary bile acid) remains the same, and that the smaller pool would be recirculated more often, the smaller pool would then contain a relatively larger proportion of the deoxycholate.

Another postulate has been brought forward implicating a change in the glycine/taurine conjugation ratio as being responsible for the lithogenic process. The glycine/taurine conjugation ratio was reported to be reduced in gallstone patients (Burnett, 1965), but this has not been generally accepted (Dam et al., 1966). Glycine conjugates are more efficient in the solubilization of cholesterol than taurine conjugates (Hegardt and Dam, 1971; Spaeth and Schneider, 1974). Schoenfield, Sjovall, and Sjovall (1966) reported that in the centers of gallstones an increase in the glycine/taurine ratio and a reduction in deoxycholate could be detected, and interpreted this as a result of an interruption of the enterohepatic circulation at the moment of stone formation. Another explanation could be that deoxycholate because of its higher solubility is not incorporated under these circumstances. To further complicate this issue Nakayama (1971) found an increase in deoxycholate in gallstones.

Bile acids are found in minute quantities in cholesterol rich stones and are the same ones as those present in bile, except that traces of two oxo bile acids have been detected, 7-ketolithocholic and

12-ketolithocholic (Izumi, 1965). Small portions of lithocholate have also been found in the centers of gallstones (Schoenfield et al., 1966). Significance of the above minor bile acids in the genesis of gallstones has not been elucidated.

Cholelithiasis is one of a group of diseases whose incidence in different countries parallels the degree of economic development. Like obesity, diabetes, coronary artery disease, and cancer of the colon, cholelithiasis is rare in Africans but increasingly common in American Blacks (Cleave, Campbell, and Painter, 1969). Clinically there are associations between gallstones and obesity, gallstones and diabetes, gallstones and coronary artery disease, obesity and diabetes, obesity and coronary artery disease, and diabetes and coronary disease (Cleave et al., 1969; Sarles et al., 1969). The more closely patients with one of these four diseases are examined, the more often they are found to have symptoms of the other three. The close associations suggest that the above mentioned diseases may have a common etiology. Several investigators have postulated this common etiology to be associated with a deficiency in dietary essential fatty acids (Hikasa, Matsuda, and Nagase, 1969) while others believe it is the increase in consumption of refined, fiber-depleated carbohydrates (Gustafsson and Norman, 1969a).

Hikasa et al. (1969) have postulated that cholesterol catabolism is impaired during cholelithiasis and have suggested that this is due to deficiency of essential fatty acids (E.F.A.). It was believed that cholesterol must be esterified with certain specific fatty acids

before being catabolized and that catabolism is slowed down if the E.F.A.s are not available for this esterification. These investigators found that adding E.F.A. rich fats to a high-sugar, fat-free diet was more effective than adding saturated fat or oleic acid-rich fat in preventing gallstone formation in hamsters (Hikasa et al., 1969). They considered that this E.F.A. deficiency is accentuated by a high intake of saturated fat, especially butter, which interferes with the activation of pyridoxal to vitamin B_6. In their animal experiments, B_6 deficiency was under some circumstances a prerequisite for stone formation. However, recently it has been shown that cholesterol can be catabolized without being esterified (Balasubramaniam, Mitropoulos, and Myant, 1975) and that gallstone patients do not show other evidence of E.F.A. or vitamin B_6 deficiency. Thus, it seems unnecessary to invoke E.F.A. or B_6 in the pathogenesis of this disease.

Gustafsson and Norman (1969b) and Heaton (1972) have compiled a considerable amount of data to indicate a correlation between the quantity of refined carbohydrate in the diet and the incidence of gallstone formation. It has been repeatedly shown in both rats (Portman and Murphy, 1958) and rabbits (Hellstrom, Sjovall, and Wigand, 1962) that on semi-synthetic diets containing a high proportion of sugar or processed starch, bile acid turnover is lower than on natural food. Likewise, Africans placed on a low-residue diet excreted less bile acids than they did on their normal high roughage diet (Antonis and Bersohn, 1962). In contrast, when young girls on a hypercholesterolemic diet were given additional dietary roughage, their bile acid pool

size increased significantly (Shurpalekar et al., 1971). These findings are further substantiated by experiments in rats which show that a diet of refined carbohydrates leads to a drastic reduction of the bile acid pool size (Lee and Herrmann, 1963; Portman, Mann, and Wysocki, 1955) and adding cellulose, Metamucil, to the diet expands the bile acid pool size (Portman and Murphy, 1958; Beher and Casazza, 1971). Although there is substantial evidence for implicating increased consumption of refined dietary carbohydrates with the incidence of gallstones, the mechanism by which refined carbohydrate alters cholesterol catabolism and bile acid pool size, beyond an influence on peristalsis and therefore enterhepatic circulation and absorption of bile acids and cholesterol within the bulk material and consequent increased fecal excretion, is not yet clearly elucidated.

Bile acids are the chief oxidative end-products of cholesterol catabolism and as previously indicated their formation, metabolism and excretion are of fundamental importance to the problem of the relationship of cholesterol to gallstone formation. Although various pathways have been worked out by observing the conversion of radiolabeled cholesterol and cholesterol metabolites to bile acids, there is little understanding of what actually regulates a number of these distinctly different pathways. It has been demonstrated that bile acids themselves feed back on the rate-limiting enzymes of cholesterol (3-hydroxymethylglutaryl CoA reductase) and bile acid (cholesterol 7α-hydroxylase) biosynthesis (Shefer, Hauser, and Mosbach, 1968; Shefer et al., 1970; Shefer et al., 1973). In addition, these investigators have concluded

that the composition of the bile acid pool influences the hepatic activity of the rate-controlling enzymes of bile acid synthesis (Shefer et al., 1973). Figure 2 illustrates the most generally accepted pathways proposed for the conversion of cholesterol to bile acids. Although only a few of the intermediates have been isolated directly from the liver or in the bile, studies of the metabolic fate of various possible intermediates have led to the proposal of a logical sequence of reactions.

The cholesterol molecule undergoes structural changes which can be generalized as either alterations of the steroid nucleus including hydroxylations or oxidation of the sidechain resulting in partial cleavage. Concerning these two general groups of reactions, far more is known about the changes involving the steroid nucleus. For instance the effects of hormones (Prager, Voight, and Hsia, 1975; Uchida et al., 1970), dietary fat (McGovern and Quackenbush, 1973), glucose (Uchida, Takeuchi, and Yamamura, 1975), individual bile acids (Shefer et al., 1973), alcohol (Lefevre, De Carli, and Lieber, 1972), and phenobarbital (Redinger and Small, 1971; Cohen et al., 1975) have been shown to influence, either directly or indirectly, reactions at the steroid nucleus. In contrast, very little is known concerning which endogenous or exogenous factors might influence the rate of cholesterol sidechain oxidation. In fact, investigators are still at odds over whether some of these reactions are mediated by microsomal (Cronholm and Johansson, 1970) or mitochondrial (Taniguchi, Hoshita, and Okuda, 1973) enzymes or whether sidechain oxidation initiates the catabolism of cholesterol

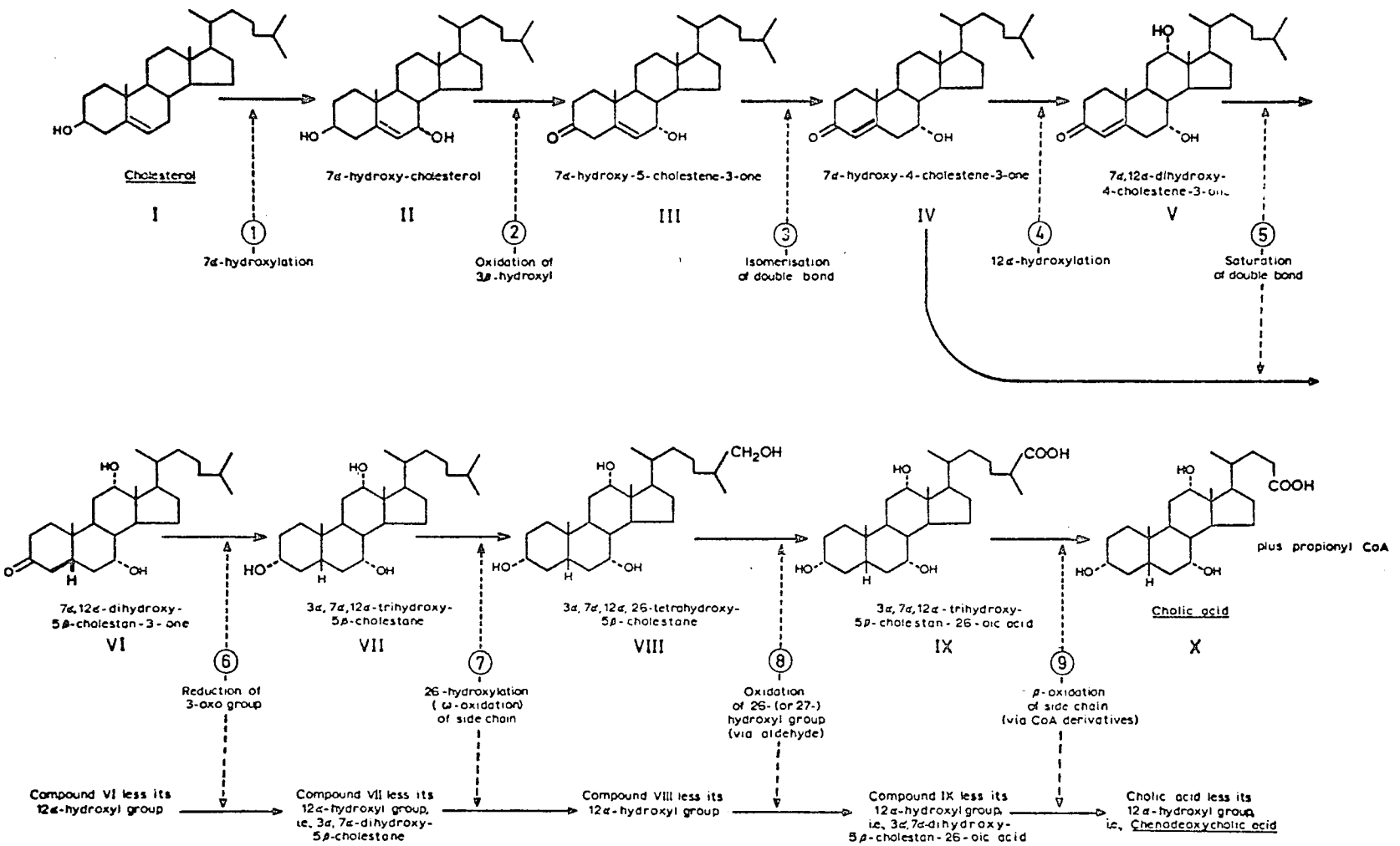

Figure 2. Proposed pathways for the synthesis of cholic and chenodeoxycholic acids from cholesterol after Heaton (1972).

Each chemical alteration is indicated by larger lettering and heavier imprinted lines.

or is only secondary following alterations to the steroid nucleus (Mitropoulos and Myant, 1969).

After investigating the metabolism of 26-hydroxycholesterol, Danielsson (1961) and later Javitt and co-workers (Javitt and Emerman, 1970; Wachtel, Emerman, and Javitt, 1968; Anderson, Kok, and Javitt, 1972) have proposed a hypothesis which states that following initial sidechain oxidation, 12α-hydroxylation of cholesterol may be restricted thus affecting the catabolic pathways and ultimately changing the individual bile acid ratios. Figure 3 illustrates this hypothetical pathway leading exclusively to chenodeoxycholic acid. These studies have made it obvious that further investigation is necessary to establish whether such a relationship exists.

Since Strand (1963) demonstrated that thyroid hormone affected an increased production of bile acids as well as a change in individual bile acid ratios, it became apparent that the thyroid hormones may be used as tools for advancing our knowledge of the individual bile acid pathways.

The following is a summary of the accumulated knowlege concerning the involvement of hepatobiliary processes in cholelitheis:

1. The basis of gallstone disease is the secretion by the liver of bile containing an excess of cholesterol in relationship to its solubilizers, bile acids and lecithin, not the malfunction of the gallbladder.

2. The incidence of cholelithiasis has been well correlated with and may have a common etiology with obesity, diabetes, and

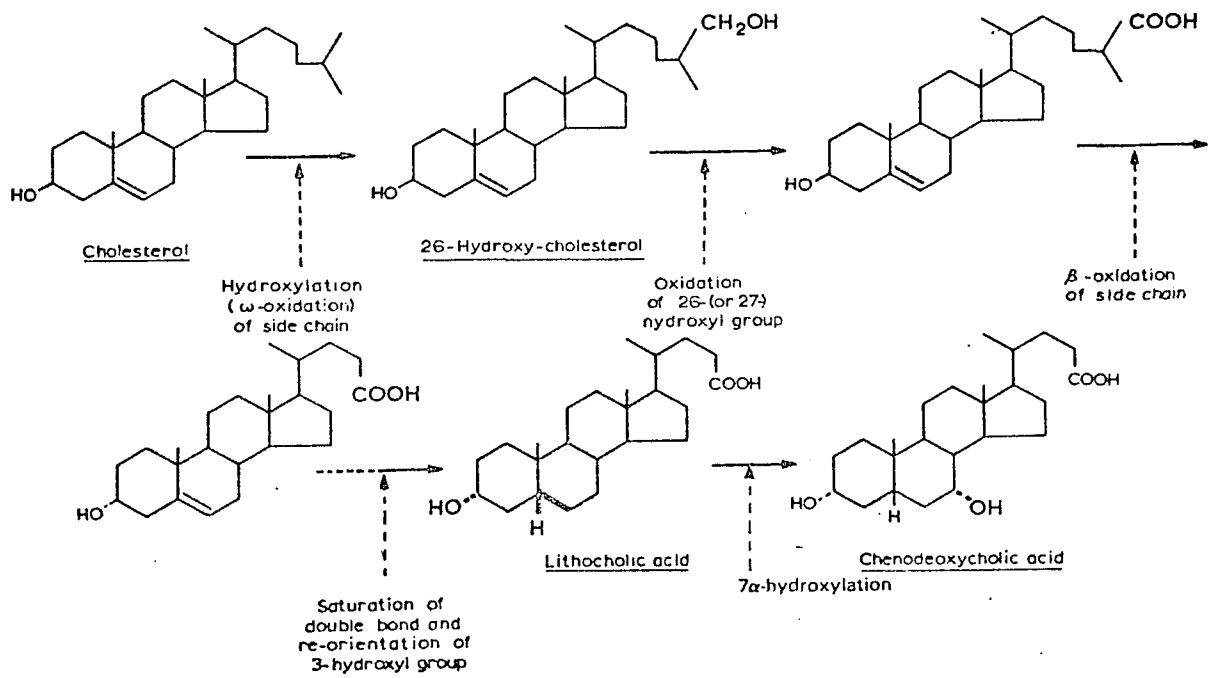

Figure 3. Postulated pathway for the synthesis of chenodeoxycholic acid following an initial oxidation of the cholesterol sidechain after Heaton (1972).

Each chemical alteration is indicated by larger lettering and heavier imprinted lines.

coronary artery disease; and it has been associated with the increased consumption of refined carbohydrates.

3. Gallstone patients have a decreased bile acid pool size.

4. Some gallstone patients have been reported to have altered trihydroxy/dihydroxy bile acid ratios as well as altered taurine/glycine conjugated bile acid ratios.

5. A depressed conversion of cholesterol to bile acids has also been noted in gallstone patients.

There is no data, however, that clearly indicate whether these observed changes are the cause or effect of the gallstone formation and which exogenous or endogenous factors (other than thyroid hormones) might be responsible for such changes.

Objectives

In order to further the understanding of the involvement of the liver in the processes of cholelithiasis it becomes important to be able to study the effects of various biochemical states produced by different substrates upon hepatic bile acid synthesis and metabolism. Thus the objective of this dissertation was to study the effects of substrates such as pyruvate, dextrose, and lactate upon synthesis and secretion of bile acids.

Since the initial investigation by Percy-Robb and Boyd (1967) demonstrating the ability of the isolated perfused rat liver to synthesize bile acids, many investigators have attempted to utilize this system in study of biliary (Boyer, 1971) and metabolic (Gonzales de Galdeano, Bressler, and Brendel, 1973; Kelman et al., 1973; Garattini,

Guaintani, and Bartosek, 1973; Noda et al., 1975) functions of the liver. However, no experiments have been performed to study the effect of different substrates on the hepatobiliary processes of bile flow, synthesis, and secretion of bile acids in this system. The isolated perfused rat liver preparation was chosen since neuronal and feed back interactions are removed thus allowing the study of purely metabolic influences on the hepatobiliary system. These experiments were amplified in certain cases by studies with isolated hepatocytes and intact rats.

In addition, to be able to facilitate the study of substrates and other pharmacological agents on cholesterol turnover to bile acids a sidechain tritiated cholesterol was synthesized. The synthesis of this compound is described and provides a new tool for measurement of the rate of cholesterol sidchain oxidation in the in vivo state as well as other in vitro systems. This method for determination of the cellular catabolic process of cholesterol turnover has the major advantage over biliary quantitation of bile acids obtained from bile in that it allows the direct determination of the catabolic process of cholesterol sidechain oxidation independent of other cellular processes such as conjugation and secretion.

CHAPTER 2

MATERIALS AND METHODS

Perfusion Experiments

Animals

The animals use for perfusion experiments were male Sprague-Dawley rats weighing 200-275 g. These animals were maintained by the Division of Animal Resources on a 12:12 light dark cycle and were fed Purina Rat Chow ad libitum. Experimentation was always begun during the first four hours of the light cycle.

Perfusion Apparatus

A perfusion apparatus was used that incorporated the following features (see Figure 4):

1) a silastic multiple tube oxygenator [1]* that could be pressurized to assure a more efficient oxygenation of the circulating buffer. The silastic tubing bundle (Dow Chemical Co.) consisted of 120 tubes of 10 inch length (0.012" i.d., 0.025" o.d.. The oxygen and carbon dioxide from the gas cylinders (indicated by the stippled arrows in Figure 4) was mixed in the gas mixing chamber [2] and forced through the silastic oxygenator at 6-9 p.s.i. which was monitored by the pressure gage [3] and adjusted by the valve [4].

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* Numbers in squared brackets refer to individual pieces of apparatus in Figure 4.

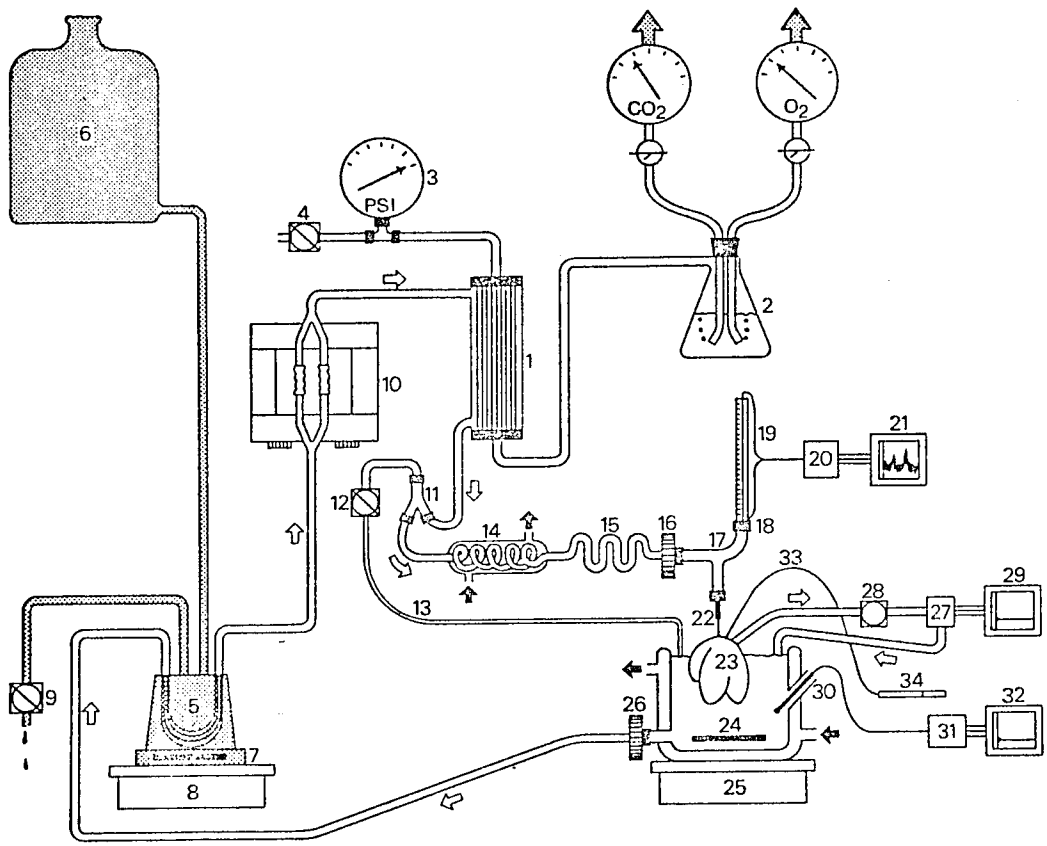


Figure 4. Apparatus for rat liver perfusions.

See text for details.

2) a hollow fiber dialysis unit [5] was employed to maintain substrate and ionic concentrations stable and remove potentially toxic metabolites. The dialysis unit was a "Bio Fiber 50 Beaker" obtained from Bio-Rad Laboratories. The flow rate of fresh buffer (stippled) from the reservoir [6] was adjusted by the valve [9] to allow an exchange of dialysate at a rate of 5 ml/min. A continual mixing of the beaker contents for adequate dialysis was assured by the magnetic stirring bar [7] operated by a stirring plate [8].

3) a Harvard Apparatus nonpulsatile perfusion pump []0] model 1210 forced the perfusion buffer through the dialysis unit and oxygenator which also served to create the pressure head at the point of the isolated liver [23]. Thus, the perfusion was by constant volume, not constant pressure. In sequence, as the oxygenated buffer ran from the oxygenator it flowed through a Y junction [11] that served as the primary bubble trap. The rate of overflow was regulated by the valve [12] which was very minimal and was returned to the general circulating pool by way of tube [13]. After buffer ran through the bubble trap [11] it was prewarmed as it moved through the heat exchanger [14]. The dark arrows correspond to the direction of prewarmed water to or from the Haake thermostatic circulating pump.

4) a flexible connection [15] to the perfusion head [17] assured freedom of movement of the perfusion head during cannulation of the <u>in situ</u> liver. To allow simplified cannulation the perfusion head was also connected by a snap-on fitting [18] to the manometer [19]. Before the buffer entered the perfusion head it was filtered through

a stainless steel syringe filter holder (25 mm, Millipore) containing a vapor flow filter (Virtis F-100 B).

5) an open manometer [19] for monitoring variations in perfusion pressure with an internal pair of electrodes was attached to a conductivity meter (Radiometer-Copenhagen CDM 2e) [20] and recorder (Bausch and Lomb VOM Linear 10) [21].

6) a short flexible line [22] led out of the perfusion head into the cannula (blunted 15 gauge needle) and from there into the liver. The buffer in the perfusion vessel [23] was maintained at 37° by the Haake pump. A magnetic stirrer [24] and stirring plate [25] was used to allow complete mixing of the outflowing perfusate with the bulk of the perfusion medium. As the buffer leaves the vessel it is filtered through another stainless steel syringe filter holder with vapor flow filter [26] to insure removal of any particulate matter released by the liver.

7) a Clark oxygen electrode recording system was used to monitor the oxygen tension of the perfusion effluent from the isolated liver. Flexible tubing used for collection purposes was tied into the vena cava. The pump [28] (Buchler Polystaltic Pump) channeled the perfusate from the vena cava into the Clark oxygen electrode [27] and returned it to the perfusion vessel. The oxygen electrode [27] monitored the levels of oxygen in the venous outflow and was attached to the Sargent recorder (Model SRG) [29].

8) a pH monitoring system with a combination glass electrode [30] was used to continuously determine the buffer pH. The pH was

maintained between 7.3 and 7.5 by altering the ratio of oxygen to carbon dioxide being forced into the circulating buffer. A pH Stat Meter PH-28 [31] and recorder [32] were used.

9) the bile duct cannula consisted of two ends of 2 cm pieces of P.E. tubing connected by a 10-15 cm piece of flexible silastic tubing (Dow Chemical Co.) [33]. One of the ends was tied into the bile duct. A holder was situated at the upper level of the liver to hold the micro pipettes [34] into which the other end of the cannula was placed and into which bile flowed from the isolated perfused rat liver.

10) an automatic lactate assay system was employed in some experiments and was connected with the venous effluent from the perfused liver. This assay system was implemented so lactate production by the liver could be continuously monitored. A proportioning pump (Technicon) sampled the perfusate directly from the vena cava at a rate of 0.6 ml/min, which was then mixed with air bubbles (one every 20 seconds) and the reagents of the Rapid Lactate Stat Pack™ (Calbiochem) at a rate of 0.1 ml/min. The reaction mixture was then pumped through a delay coil (reaction time of 15 minutes) at the end of which the bubbles were removed. The bubble free reaction mixture then entered the flow cell (Helma 178) in the spectrophotometer (Gilford 240) and the optical density was recorded (Sargent Recorder SRG).

Perfusion Buffer

A modified Krebs Henseleit buffer containing 2% PVP (polyvinyl pyrrolidone, pharmaceutical grade, Sigma Chemical Co.) was used for the liver perfusions. The buffer contained 119 mM NaCl, 4.7 mM

KCl, 1.2 mM KH_2PO_4, 24.5 mM $NaHCO_3$, 3.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.3 μM EDTA, 60 μg/ml potassium penicillin (E. R. Squibb and Sons, Inc.) and 10 mM pyruvate (sodium salt, Calbiochem), 10 mM lactate (L +, 30% aqueous solution, Sigma Chemical Co.) or 10 mM dextrose (U.S.P. powder, Mallinckrodt Chemicals) as indicated. Before the buffer was employed in the perfusion it was filtered through a Millipore filter of pore size 0.45 μ and adjusted to pH 7.4-7.6 with 0.1 N NaOH. All chemicals used were of analytical reagent grade.

Surgical Procedure

Rats were anesthetized and maintained under ether throughout surgery. An abdominal incision was made along the midline and extended bilaterally. The bile duct was cannulated close to the liver with the modidied P.E. 10 silastic tubing [33]. The cannula of the perfusion apparatus (grooved 15 gauge stainless steel needle) was inserted and tied into the portal vein in the general locus of the lienal branch. The vena cava was opened at the level of the right kidney, and perfusion was started while the animal was still alive. The perfusing liver was surgically removed from the animal, trimmed of any extraneous tissue, washed out with 200 ml fresh buffer and placed into the perfusion vessel. The entire surgical procedure took an average of 10 minutes with an ischemic period of less than 30 seconds.

Liver Function Tests

Bile Flow Determination

Bile flow was determined by observing the time required to collect 50 or 100 µl of bile in calibrated "Dade" disposable micro pipettes. Bile flow rates were computed as µl bile formation per minute.

Sodium - Potassium Determination

Sodium and potassium concentrations in 20 µl bile samples were determined in an IL Model 143 flame photometer in 4 ml lithium diluant. A 140 mEq/liter [Na^+] - 5 mEq/liter [K^+] solution was used to standardize the flame phctometer. Sodium secretion rate was calculated as mEq/min. Potassium secretion rate was also calculated in mEq/min.

Glutamic Oxaloacetate Transaminase Determination

Glutamic oxaloacetate transaminase (GOT) activity was measured intermittantly in 0.5 ml of circulating perfusion medium by a manual UV assay method described by Bergmeyer and Bernt (1968). The reaction sequence

$$\text{L-Aspartate} + \text{2-Oxoglutarate} \underset{}{\overset{\text{GOT}}{\rightleftharpoons}} \text{Oxaloacetate} + \text{Glutamate} \qquad (1)$$

$$\text{Oxaloacetate} + \text{NADH} + H^+ \overset{\text{MDH}}{\rightleftharpoons} \text{L-Malate} + NAD^+ \qquad (2)$$

illustrates the basis for the GOT determination. As indicated, NAD is an end-product of the respective reactions being stoichiometrically equal to the quantity of GOT activity in the sample and can easily

be determined by spectrophotometry. The equilibrium lies far to the right in the first reaction (Green, Leloir, and Nocito, 1945). The oxaloacetate formed reacts immediately and therefore the GOT activity is determined by the rate of NADH oxidation in the second reaction.

The following reagents, potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), L-aspartate (potassium salt, Sigma Chemical Co.), 2-oxoglutaric acid (dipotassium salt, Sigma Chemical Co.), reduced nicotinamide adenine dinucleotide (NADH, Boehringer Mannheim GmbH), malate dehydrogenase (MDH, Boehringer Mannheim GmbH), lactate dehydrogenase (LDH, Boehringer Mannheim GmbH), sodium hydroxide, glycerol, and sodium hydrogen carbonate, were acquired for use in the GOT determination. All chemicals were analytical reagent quality.

The following solutions, a phosphate/aspartate solution (0.1 M phosphate buffer of pH 7.4, 0.25 M aspartate), a NADH solution (13-15 mM), a MDH/LDH solution (the commercial enzyme solutions were mixed with 50% glycerol to give 0.5 mg of each protein/ml), and a 2-oxoglutarate solution (approximately 0.45 M and adjusted to pH 7.0 with 5 N NaOH), were made up fresh every two weeks.

The assay was performed using a Gilford 240 Spectrophotometer set at 340 nm with a light path of 1 cm at room temperature. To each cuvette 3.0 ml of the phosphate solution, 0.05 ml of the NADH solution, 0.05 ml of the MDH/LDH solution, and 0.5 ml of sample perfusion buffer was added, mixed and allowed to stand for 5 minutes. After the preliminary incubation 0.1 ml of 2-oxoglutarate was added after which the

GOT reaction occurred. The cuvette contents were mixed, the extinction read against an air blank and a stop watch was started. Readings were taken after exactly 1, 2, and 3 minutes and were followed by calculating the mean extinction differences. Enzyme activity in the samples were determined by using the following equation,

$$\text{activity in sample} = \frac{1000}{e \times d} \times \frac{\Delta E}{\Delta t} \text{ in units per liter,}$$

where e is the extinction coefficient, d is the light path in cm, Δ E is the change in extinction, and Δ t is the interval between measurements in minutes.

Lactate Dehydrogenase Determination

In addition to GOT, lactate dehydrogenase (LDH) was also intermittantly measured as described by Bergmeyer and Bernt (1968) in 0.5 ml samples of circulating perfusion medium. The reaction

$$\text{Pyruvate} + \text{NADH} + H^+ \underset{\longleftarrow}{\xrightarrow{\text{LDH}}} \text{L-Lactate} + \text{NAD} \qquad (3)$$

demonstrates the basis for the LDH determination. As is indicated, the NAD produced can be quantitively measured by spectrophotometry and correlates with the activity of LDH found in the sample.

The following reagents, potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), pyruvate (sodium salt, Calbiochem), reduced nicotinamide adenine dinucleotide (NADH,

Boehringer Mannheim GmbH) and sodium bicarbonate, were obtained . All chemicals were analytical reagent quality or better.

A phosphate/pyruvate solution (50 mM phosphate, pH 7.5, 0.63 mM pyruvate) and a nicotinamide-adenine dinucleotide solution (approximately 11 mM in 10 mM $NaHCO_3$) were prepared fresh each week.

The assay procedure was performed using a Gilford 240 Spectrophotometer set at 340 nm with a light path of 1 cm at room temperature. To each cuvette 3.0 ml of the phosphate/pyruvate solution was pipetted, along with 0.05 ml of the NADH solution, plus the 0.5 ml of perfusion medium sample. The contents of the cuvette were mixed and the extinction read immediately and subsequently at 1, 2, and 3 minutes. The mean change in extinction can be calculated and the LDH activity in the samples determined by using the same equation described in the previous section for determining GOT activity in the perfusate.

Lactate Determination

In selected experiments (in which exogenous substrate was supplied only as dextrose) lactate levels were measured in an attempt to determine if a relationship exists between hepato-vascular tonus and lactic acid formation by the liver. In order to detect small as well as discrete changes in lactate concentrations a continuous and rapid assay system was employed. The following reactions

$$\text{L-Lactate} + \text{NAD} \xrightleftharpoons{\text{LDH}} \text{Pyruvate} + \text{NADH} \qquad (4)$$

$$\text{Pyruvate} + \text{L-Glutamine} \xrightleftharpoons{\text{GPT}} \text{L-Alanine} + \alpha\text{-ketoglutarate} \qquad (5)$$

illustrate the basis for the lactate assay used. In reaction (4) the NAD before reduction would be colorless at 340 nm, and thus the quantity of lactate reacting with LDH produces an equal quantity of NADH which is visible at 340 nm and can be quantitatively determined by spectrophotometry. In reaction (5), glutamic pyruvate transaminase (GPT) serves to trap enzymatically the pyruvate formed. Through employing an enzymatic trapping agent in place of commonly used chemical reagents, the velocity of the reaction is increased.

The Rapid Lactate Stat-Pack™ (Calbiochem) was used for making up the necessary reagents because of the convience of preparation and the decreased reaction time required. Vial A and B of the Stat-Pack™ were mixed with 15 ml distilled water and used as the reaction reagent in the automated apparatus as previously described under the heading Perfusion Experiments.

Analytical Procedures

Total Bile Acid Determination in Bile

The total bile acids secreted into 50 µl bile samples were determined by the modified enzymatic 3α-hydroxysteroid dehydrogenase method of Iwata and Yamasabi (1964). The major bile acids are 24 carbon steroids with a common 3α-hydroxy moiety which is oxidized by a NAD-dependent 3α-hydroxysteroid dehydrogenase prepared from Pseudomonas Testosteroni to produce NADH that can be determined spectrophotometrically. The enzyme reaction used for the determination of total bile acids in bile samples from isolated perfused livers was the following:

$$\text{Bile Acids} + \text{NAD} \xrightarrow{3\alpha\text{SDH}} \text{3-Keto-Bile Acids} + \text{NADH}. \qquad (6)$$

The following reagents, glycine (crystalline), hydrazine sulfate, EDTA (tetrasodium salt), nicotinamide-adenine dinucleotide (NAD, Boehringer Mannheim GmbH), tris (hydroxymethyl) aminomethane, 3α-hydroxysteroid dehydrogenase (Worthington Biochemical Corp., one unit of activity equals 1 μM of substrate oxidized per minute in the presence of NAD of pH 8.9 at 25°), sodium hydroxide, conjugated (taurine) and unconjugated cholic and chenodeoxycholic acids (Supelco, Inc.), were obtained.

A hydrazine/glycine solution with EDTA (0.8 M hydrazine sulfate, 1.9 M glycine, 11 mM EDTA) - 3 parts, was mixed with 3 parts of 2 N NaOH and 4 parts of water. A 5 mM solution of NAD and an enzyme solution (two mg enzyme in one ml of 0.03 M Tris buffer at pH 7.2), as well as a standard mixture of taurocholic (40%), taurochenodeoxycholic acid (40%), cholic acid (10%), and chenodeoxycholic acid (10%) were prepared fresh every week.

The total bile acid concentration was measured using a Beckman Acta V, dual beam spectrophotometer equipped with an automated carriage sampler unit and time plotter system. Four samples were run at a time, each in an individual cuvette, continuously electronically subtracting for the blank. I found this procedure to be far superior to recording incubation times because after adding the enzyme, the blank was found to increase in optical density (O.D.). Investigators

have tried to stabilize the blank by boiling the enzyme before adding it to the blank or by removing the NAD from the blank. Using this automated system, a better quantitation is obtained since the reaction kinetics are plotted out for each sample. The blank is a real control blank, and the standards are more reproducible from day to day. In the procedure a 50 µl bile sample was pipetted into a clean cuvette, a 750 µl portion of combination hydrazine/glycine buffer followed by 100 µl of NAD solution was then added to the cuvette. The cuvettes are all blanked and zeroed on the plotter after the contents of each cuvette were mixed and allowed to stabilize. The enzyme solution (20 µl) was then added as quickly as possible to all five cuvettes, mixed again, the automatic sampler started and then allowed to plot the development of O.D. at 340 nm (NADH formation) for 15 minutes. As a control for the enzymatic determination of total bile acid concentration, a known amount of the standard bile acid solution was always assayed in conjunction with each series of determinations. Figure 5 illustrates a series of bile sample analyses and also a standard curve used for quantitation purposes. The total bile acid concentration was calculated as mg bile acids in 50 µl. The total bile acid secretion rate was figured as the bile acid concentration secreted per minute.

Individual Bile Acid Determination
by GLC and Mass Spectrometry

To hand-blown microbulbs (Figure 6) 50 µl of bile sample and 100 µl of 2 N NaOH was added, quick frozen and flame sealed. The samples were then hydrolyzed for 3 hours in the autoclave at 15 psi at

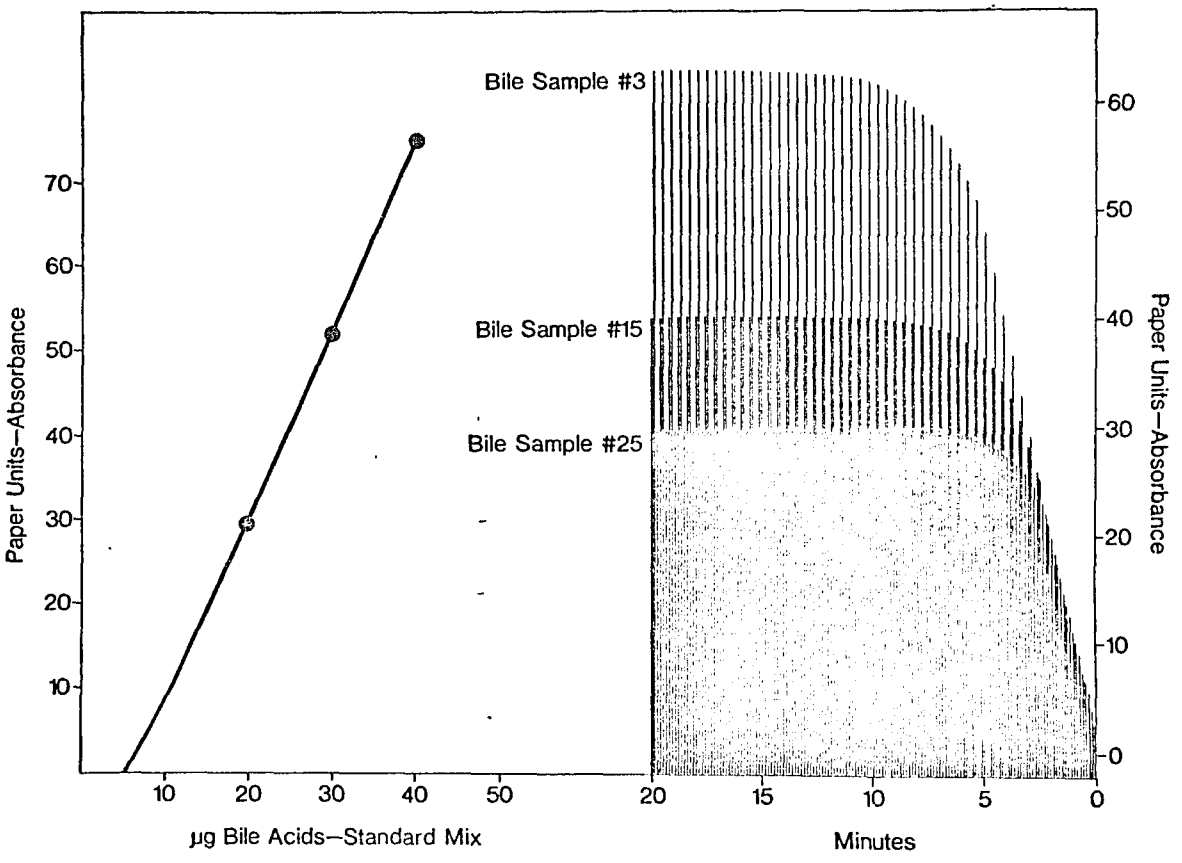


Figure 5. Total bile acid quantitation of standard concentrations and unknown bile samples obtained from perfused livers.

A. A standard curve was generated by analyzing 20, 30, and 40 µg respectively of the standard bile acid mixture containing 40% taurocholic, 40% taurochenodeoxycholic acid, 10% free cholic acid, and 10% free chenodeoxycholic acid.
B. Reaction kinetics for three bile samples collected at 26, 128, and 261 minutes respectively from an isolated perfused rat liver. Total bile acid secretion rate in µg/min. is determined from the standard curve A.

Figure 6. A sealed hand-blown microbulb containing a 50 µl bile sample and sodium hydroxide solution.

120° C. The microbulbs were then crushed with a glass rod at the bottom of 13 ml conical centrifuge tubes with screw caps lined with Teflon, followed by rinsing the stirring rod with 1 ml 1 N NaOH. The mixtures of base and broken up glass were extracted with 5 ml ether and subsequently transferred to 15 ml concical centrifuge tubes. The ether extracts were evaporated to dryness after each extraction. The residue was dissolved in 0.5 ml methanol and then 0.5 ml of freshly prepared diazomethane ether solution was added to each tube (Klaassen, 1971). Fifteen minutes after addition of the diazomethane, the solvent was removed by placing the tubes in a warm water bath and blowing dry nitrogen into each sample tube. It was imperative that the samples were absolutely dry before derivatization. The trifluoroacetates of the bile acid methyl esters were prepared by the addition of 0.5 ml freshly distilled trifluoroacetic amhydride (Matheson Coleman and Bell). The tubes were then capped with teflon lined screw caps and allowed to react at 37° for 30 minutes, and then evaporated to dryness. The residue was taken up in 50 µl of chloroform (redistilled) of which 5-10 µl was injected on a three foot glass column - 6.5 mm O.D., 2 mm I.D. which was packed with 1.5% QF-1 on 80/100 mesh Chromosorb W mounted in

a Hewlett Packard 5700 chromatograph equipped with automatic sampler and intergrater. Elution and desired separation was achieved utilizing the following temperature program: 4 minutes isolthermal at 130° C, 35 minutes 130-240° C., 8 minutes isothermal at 240° C.

Since the first demonstration by Vanden Heavel, Swelley and Horning (1960) that bile acid methyl esters can be separated by gas-liquid chromatography (GLC) this technique has become one of the most important methods for qualitative and quantitative analysis of bile acids (Sjovall, 1964; Kuksis, 1966; Grundy, Ahrens, and Miettinen, 1965). The most important recent development in this field is the construction of gas chromatography-mass spectrometry (GC-MS) instruments suitable for analysis and structural determination of high molecular weight compounds such as bile acids. Several GLC peaks observed in the bile samples from the isolated perfused livers could not be identified and prompted me to use GC-MS analysis for identification of the unknown peaks as well as for positive confirmation of the additional peaks identified by comparison with the relative retention times of known bile acid standards. In these studies a Finnigan 3300-6100 quadrupole mass spectrometer with data system was utilized in conjunction with the Finnigan GLC run isothermal with helium flow rate of 20 ml/minute.

Analysis Procedure for Determining
Cholesterol Sidechain Oxidation Rate

Sidechain oxidation of [24,25-^{3}H]-cholesterol yields tritiated water as explained in more detail in the Result and Discussion section. The tritiated water formed equilibrates throughout the cellular as well as extracellular water of the preparation.

Samples of 500 µl of perfusate were taken at various times from the circulating medium and placed on top of a charcoal column (approximately 2 inches in disposable Pasteur pipettes) prepared from a slurry of activated charcoal (Darco G-60, Matheson Coleman and Bell), 1:5 charcoal to water. These glass charcoal columns were set in rubber stoppers (number 2) which fit directly into the tops of scintillation counting vials (see Figure 7). These precounted vials contained 7 ml

Figure 7. Illustrated charcoal column and scintillation vial set up for separating $^{3}H_{2}O$ from [24,25-^{3}H]-cholesterol in samples.

counting solution. The scintillation cocktail, one part of Triton X-100 (Rohm and Haas) to two parts of toluene (Matheson Coleman and Bell) with 3.8 g/liter of Omnifluor (New England Nuclear Corp.), was prepared. After loading the samples on top of the charcoal columns, the column vial aggregates were spun at 45 x G for 10 minutes in a swinging bucket International Centrifuge. The columns were then washed twice with 1 ml of water each to elute all tritiated water by centrifugation through the charcoal into the scintillation counting cocktail, while radioactive cholesterol and its metabolites remain absorbed to the charcoal. The vials were then taken from the centrifuge, the column stoppers were removed, and an additional 10 ml cocktail added. The mixture was allowed to equilibrate in the dark for 3 hours and was then counted in a Beckman LS-250 liquid scintillation counter. The charcoal procedure was also applied to the incubation medium of isolated hepatocytes with minor alterations. Although the charcoal columns were required for separating out the large quantity of radioactive cholesterol from a smaller proportion of ^3H_2O, an alternative method was also used for the perfused livers and urines from the experiments with intact animals. Thus whenever applicable, the charcoal columns were replaced by columns of the same dimensions of Dowex 50 x 8 (200-400 mesh) or by mixed bed resin columns made up by adding equal amounts of Dowex 50 x 8 (H^+ form, 200-400 mesh) and Dowex 1 x 8 (OH^- form, 200-400 mesh). The advantage of this modification is a faster process of the separation-elution, since centrifugation was not necessary, but the disadvantage is the limited absorption capacity for the radioactive cholesterol and its metabolites.

Synthesis of [24,25-^{3}H]-Cholesterol

The procedure described is similar to that published for the conversion of stigmasterol to β-sitosterol (Steele and Mosettig, 1963).

Desmosterol Tosylate Formation

To a "miniactor" (Applied Science Laboratories, Inc.) 100 mg desmosterol (Steraloids, Inc.) and 300 mg p-toluenesulfonly chloride which was freshly recrystallized was added and dissolved in 0.5 ml dry pyridine freshly distilled over CaH_2. The reaction mixture was allowed to stand overnight at room temperature and in the dark. The reaction mixture was then poured into 25 ml ice cold saturated potassium bicarbonate solution. The precipitated tosylate was then filtered and washed carefully with a 1:1 acetone to water solution. A small portion of the orange-white material was dissolved in ether and run on thin layer chromatography (TLC) plates (Pre-coated 0.25 mm Silica Gel 60, E. Merck AG-EM Laboratories, Inc.) with chloroform as the solvent to identify the products and determine the extent of desmosterol tosylate formation (Figure 8). Figure 9 illustrates the structural changes produced during the synthesis of [24,25-^{3}H]-cholesterol (VII) from desmosterol (I).

Iso-Desmosterol Formation

After dissolving approximately 100-125 mg of the crude desmosterol tosylate obtained in the previous step in 30 ml of acetone, 48 mg of potassium bicarbonate in 3 ml of distilled water was added, the mixture refluxed for 6 hours and then evaporated down to 1/3 of its volumn. The remaining mixture was diluted with water until no further amount of

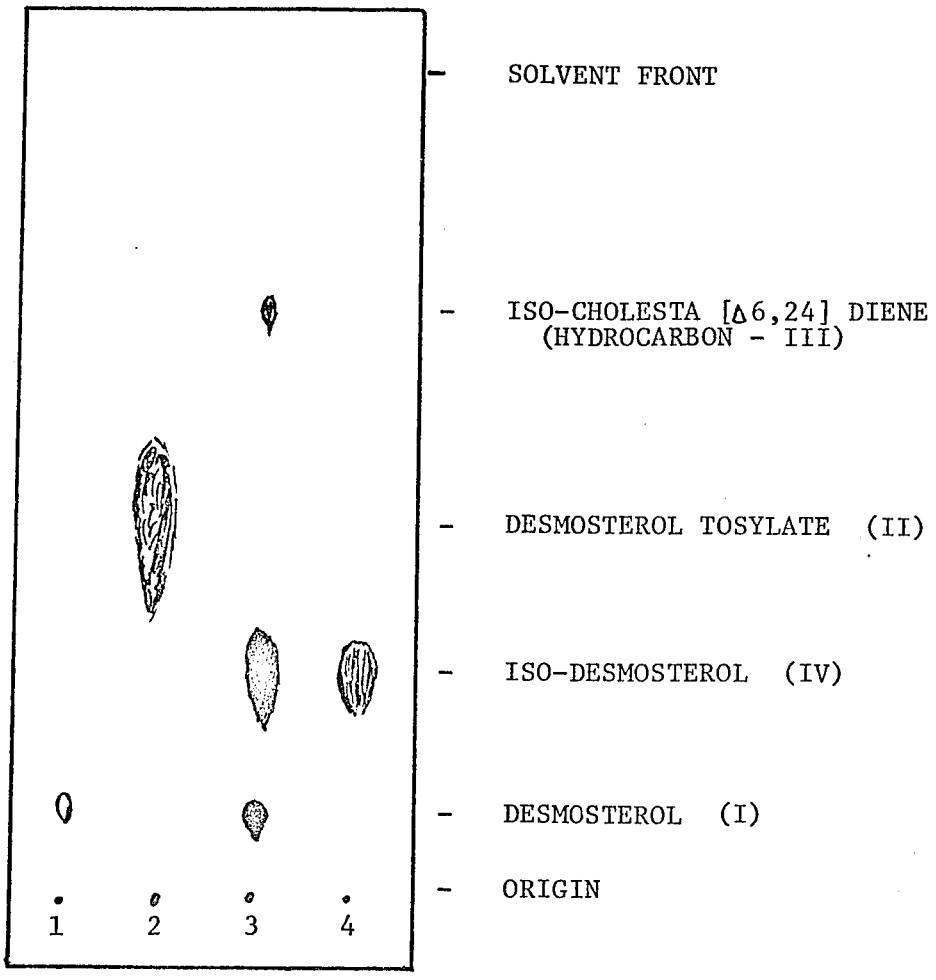


Figure 8. Thin layer chromatography determination and separation of the various intermediate reaction products.

1- Desmosterol standard
2- Reaction mixture of desmosterol and p-toluene sulfonyl chloride
3- Solvolysis products of desmosterol tosylate
4- Eluate fraction from silicic acid column containing iso-desmosterol- separated from other solvolysis products

Note- this solvent system, chloroform, did not separate desmosterol from cholesterol or iso-desmosterol from iso-cholesterol.

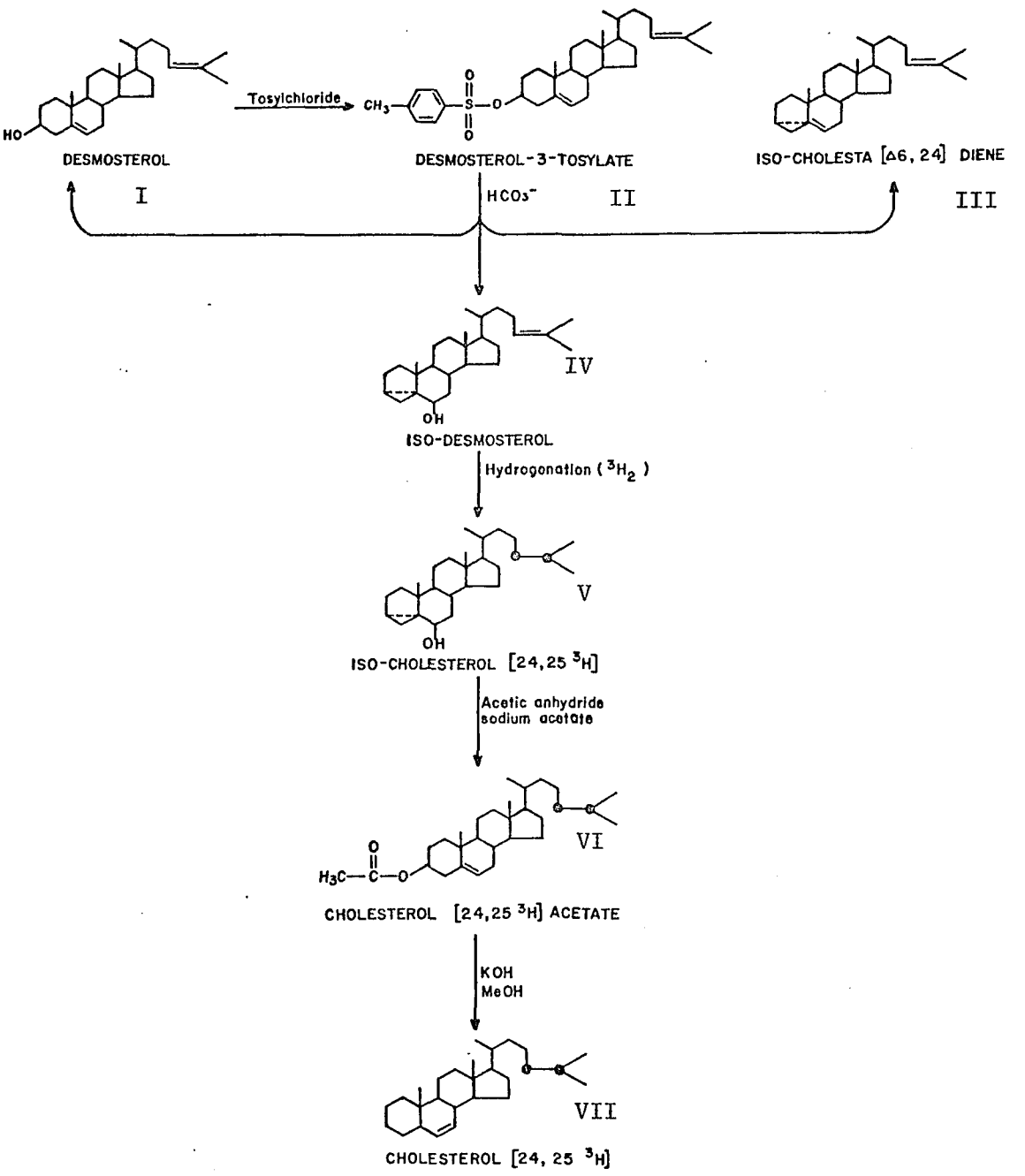


Figure 9. Synthesis of [24,25-^{3}H]-cholesterol from desmosterol.

precipitation was apparent, and then extracted with ether. The ether extract was washed with water, dried over anhydrous potassium carbonate and evaporated to dryness yielding a faint yellow oil which was determined by TLC to be a combination of desmosterol (I), iso-desmosterol (IV), and hydrocarbon (iso-cholesta-[6,24]-diene, III) (Figure 8). The iso-desmosterol (IV) was separated and purified by placing the yellow oil dissolved in hexane on a 7.5 g column of silicic acid (minus 320 mesh, Bio Rad Laboratories), and eluting the column as follows: 40 ml of hexane, fraction 1-20; 40 ml hexane/benzene (1:1), fractions 21-40; 40 ml hexane/benzene (2:3), fractions 41-60; 40 ml chloroform, fractions 61-80. Figure 10 depicts the elution of the solvolysis products from the silicic acid column when following through the procedure with a tracer portion of [26-^{14}C]-desmosterol (New England Nuclear). The separation of the iso-desmosterol (IV) in fractions 41-60 from the other solvolysis products by the silicic acid column is shown in Figure 10.

An important modification of the Steele and Mosettig (1963) method was the use of silicic acid instead of florisil. These authors used florisil which is a very inactive adsorbant, to prevent the occurrence of iso-desmosterol (IV) breakdown to the hydrocarbon (III). I found however, that florisil was very inefficient for the separation and that after careful washing with water, methanol, and hexane, the silicic acid columns separated the solvolysis products significantly better and caused a minimum of hydrocarbon formation. The fractions containing the iso-desmosterol were pooled and evaporated to dryness leaving an oil which crystallized from acetone-methanol to give colorless crystals.

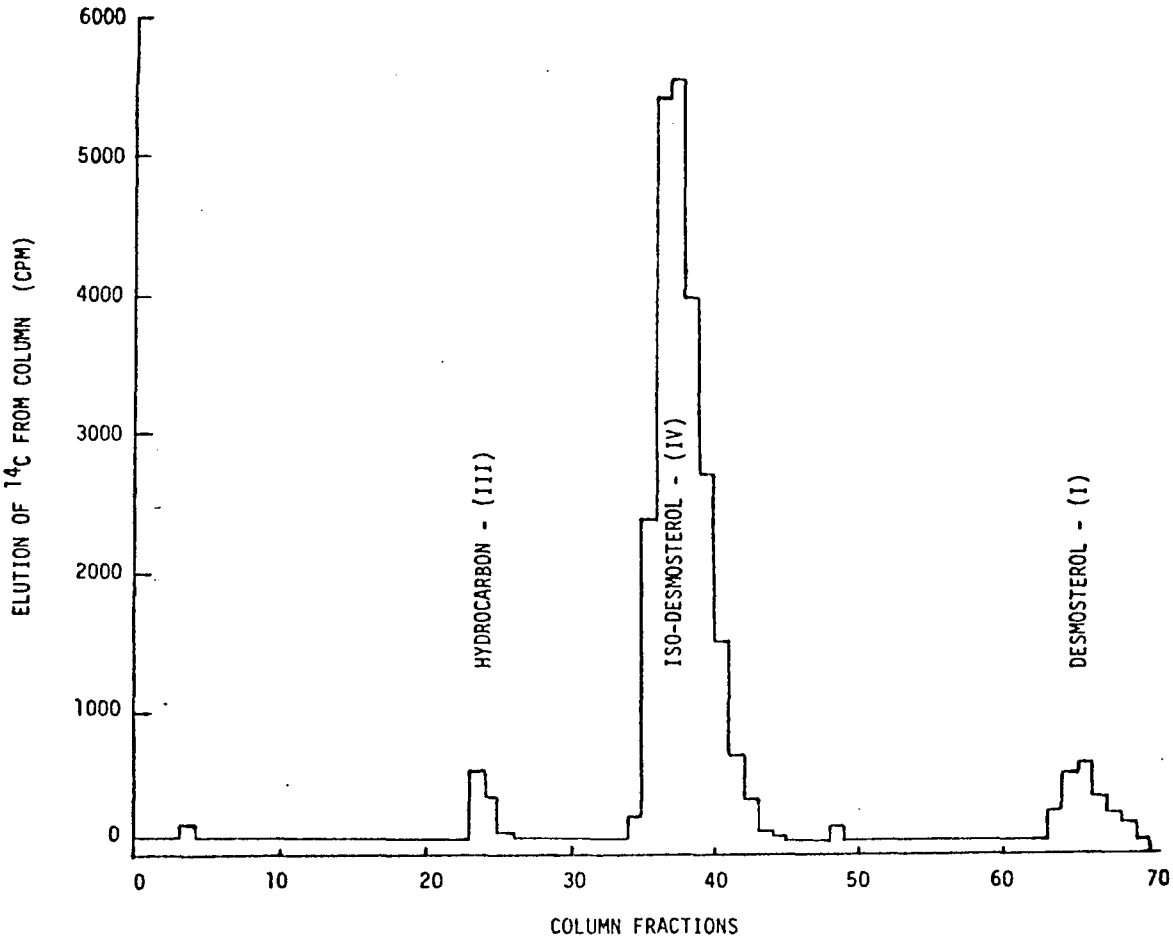


Figure 10. Elution of [26-^{14}C]-desmosterol tosylate solvolysis reaction products from a silicic acid column.

Fractions: 1-20 hexane
21-40 hexane/benzene (1:1)
41-60 hexane/benzene (2:3)
61-80 chloroform

Each fraction represented 2 ml eluate collection from the 7.5 g silicic acid column.

[24,25-^{3}H]-Iso-Cholesterol Formation

A special 1.5 ml reaction vessel was prepared with a silicon rubber septum and side-arm incorporating microstirring bar (Figure 11).

Figure 11. Specially prepared reaction vessel.

This vessel was sent to New England Nuclear (Boston) to be filled with carrier-free tritium gas and then vacuum sealed. Upon return to the laboratory a solution of 0.5 ml ethyl acetate and 5% palladium on charcoal was carefully injected into the vial. The recrystallized isodesmosterol (IV), after being dissolved in 0.5 ml ethyl acetate, was also injected into the reaction vessel and allowed to stir overnight. The reaction was then completed by injecting $^{2}H_{2}$ and further stirring for 5 hours. The entire liquid content was then withdrawn from the vessel, extracted with ether, and evaporated to dryness. A small portion of the white residue was taken up in ether and mixed with material which had been obtained in a previous run in which hydrogen was used instead of the tritium. This material was then plated on silica TLC plates impregnated with $AgNO_{3}$ (Silica Gel H-ADN, 20% impregnation,

Applied Science Laboratories, Inc.) and shown to be a single spot different from desmosterol, iso-desmosterol, cholesterol and the hydrocarbon (III). A total of 93% of the radioactivity was found in the spot corresponding to [24,25-^{3}H]-iso-cholesterol.

[24,25-^{3}H]-Cholesterol Acetate Formation

The product from the previous step was refluxed with magnetic stirring for 6 hours in 2 ml acetic anhydride, 0.3 ml acetic acid, and 45 mg potassium acetate. The mixture was diluted with water, cooled to 0° C., and filtered leaving approximately 30-50 mg [24,25-^{3}H]-cholesterol acetate (VI) as tiny white crystals. To insure stability of label and pruity of compound, an equal portion in mg of cold cholesterol acetate (99+% chromatographic pure, Sigma Chemical Co.) was added and followed by recrystallization 3 times from methanol after which the specific activity remained constant.

[24,25-^{3}H]-Cholesterol Formation

Hydrolysis of the [24,25-^{3}H]-cholesterol acetate (VI) with 5% methanolic potassium hydroxide gave [24,25-^{3}H]-cholesterol (VII) with a specific activity of 14.1 mCi/mM which was not altered by further recrystallization.

Isolated Suspended Hepatocytes

Animals

Male Sprague-Dawley rats were maintained on an alternating 12:12 day-night light cycle fed Purina rat chow ad libitum.

Buffers

Due to the complexity of isolation and sensitivity of the liver cells, the following buffers were required for isolation purposes:

1) Perfusion Buffer- 3% dextran T-70 (Pharmacia), 69 mM NaCl, 71.6 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Sigma Chemical Co.), 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.3 mM $MgSO_4 \cdot 7\ H_2O$, 5.3 µM EDTA (ethylenediamine tetracetic acid, Sigma Chemical, Co.), and 60 µg/ml potassium penicillin (E. R. Squibb and Sons, Inc.).

2) Wash Buffer- 3% dextran T-70, 130 mM NaCl, 10 mM HEPES, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.3 mM $MgSO_4 \cdot 7\ H_2O$, 5.3 µM EDTA, and 60 µg/ml potassium penicillin.

3) Incubation Buffer- 10 mM L-lactic acid (Sigma Chemical Co.) or sodium pyruvate (Calbiochem), 0.5 ml 0.5 ml 0.154 M $CaCl_2$, 1 ml amino acid mixture (100 X- Grand Island Biological Company- GIBCO), 1 ml L-glutamine (200 mM, GIBCO) was made up to 100 ml with perfusion buffer without EDTA with a final concentration of 2% albumin (Fraction V, Sigma Chemical Co.). All buffers were adjusted to a final pH of 7.4 and filtered through a Millipore filter of 0.45 micron pore size prior to use.

Perfusion System

This perfusion system is similar to that already described, but the apparatus is simplified, consisting only of the following: isolated organ in vitro; a circulating perfusate volume of 100 ml; an enclosed silastic membrane oxygenating system; a constant volume non-pulsatile pump; perfusion pressure monitoring; the thermostated perfusion vessel with the canulated organ submerged inside.

Preparation of Hepatocytes

The initial phase in preparation of the hepatocytes is essentially the same procedure as for perfused liver preparations. In short, the rats are surgically opened as described previously, portal vein canulated and approximately 25 ml of perfusate were flushed through the organ before the liver was transferred from the abdominal cavity to the perfusion apparatus. The perfusion apparatus was filled prior to the liver isolation with 100 ml of the perfusion buffer, and allowed to equilibrate with oxygen at 32°. Collagenase (132 units/mg, Worthington Biochemical Corp.) of 50 mg in 25 ml perfusion buffer was then added to the recirculating perfusate. The perfusion time in the presence of collagenase was 15-20 minutes at a flow rate of 10 ml per minute. When the liver had significantly increased in size and started to leak perfusate out of the lobes, the system was changed to non-recirculating and the collagenase-containing buffer washed out of the liver. Three washes of buffer (50 ml each) were perfused through the liver to wash out the remaining collagenase. The liver was disconnected from the cannula, the tissue gently separated by gentle opening of scissors, and poured through a sieve of 1 mm mesh size. The liver was washed through this sieve into a second plastic beaker with bovine serum and the gentle stirring motion of a soft rubber pipette bulb. Fresh cow blood was obtained from a nearby slaughter house, it was allowed to clot, then centrifuged, and the serum decanted and kept frozen at -30°. The bovine serum contains an α_2-macroglobulin which irreversibly binds to and inhibits collagenase (Webb et al., 1974), and thereby helps remove the

enzyme activity. The crude liver cell suspension was drawn into a 100 ml pipette through a 3 mm fire-polished opening. This procedure was repeated two times to dispense the cells. The suspension was then placed in the top conical tube of two stacked 50 ml plastic centrifuge tubes. The bottom tube was prefilled with 45 ml clean wash buffer and the upper tube was a conical centrifuge tube with a 6 mm opening in the bottom. The tubes were centrifuged two minutes at 40 x G, forming a loosely packed pellet. The supernatant was discarded and the pellet was resuspended in wash buffer, and the washing procedure repeated 2 additional times. The final pellet was resuspended in 100 ml of the incubation buffer.

To assess the cell viability following this technique the trypan-blue exclusion test using 0.25% trypan blue in the incubation medium indicated that 95% of the cells remained intact.

Incubation Conditions

Each incubation reaction contained 5 ml cells which were pipetted into specially prepared 25 ml flasks according to Hayes and Brendel (1976). The concentration was 45-60 mg wet weight cells per ml or about 4.4 to 5.9 x 10^6 cells per ml. All incubations were carried out at 30°. The reaction flasks were swirled in a gyrating incubator at 80 revolutions per minute.

Analysis Procedure for Determing the Rate of Cholesterol Sidechain Oxidation *In Vitro*

Samples of 500 µl of hepatocyte suspension were taken from the individual incubation flasks at the indicated times and placed in small

1.5 ml conical centrifuge tubes (Brinkmann). These suspensions were separated by a 15 second centrifugation in a Brinkmann Microfuge. A 300 µl portion of supernatant was taken from each centrifuge tube and placed on charcoal columns as described previously. The eluate was counted after equilibration in the dark and the rates of tritiated water formation which equaled the rate of cholesterol sidechain oxidation was calculated from the increase of counts as incubation time proceeded.

In Vivo Experimentation

Animals and Experimental Procedure

All animals were Sprague Dawley rats obtained from Charles River of California weighing between 150 and 250 grams. Some of the litermates were thyroidectomized before shipment. One week was allowed after arrival for acclimation before any treatment or experimentation was begun. All animals received Dialyte (Abbott Laboratories), a lactate, dextrose, salt solution as drinking water supplement.

Four groups of animals were tested: 1) D-thyroxine (400 µg/kg per day) treated animals, 2) L-thyroxine (100 µg/kg per day) treated animals, 3) thyroidectomized animals, and 4) control animals injected with 0.9% saline solution each day. Each group of animals was treated for one week with the respective treatment described prior to experimentation. After one week the radioactive cholesterol was administered to each animal. The animals were then placed in metabolic cages (Econo Metabolism Unit, Scientific Products) for the entire experimental period of 12 days. The respective treatments to each animal group was further

continued over the entire experimental period. All urine samples were measured in volumn and collected at 12 hour intervals.

Analysis Procedure for Determing the Rate of Cholesterol Sidechain Oxidation *In Vivo*

From each 12 hour urine sample, one ml was taken and run down a mixed bed resin column of Dowex 1 x 8 hydroxyl form and Dowex 50-W x 8 hydrogen form, to insure that all counts observed were indeed $^{3}H_{2}0$, and secondly, to remove the urinary salts and color for more efficient liquid scintillation counting. An additional two ml of water were used to wash the resin of all $^{3}H_{2}0$ into the counting cocktail.

CHAPTER 3

RESULTS AND DISCUSSION

Initial experimentation, utilizing a relatively simple liver perfusion system without a dialysis unit, was carried out with buffer exchanges every 45 to 60 minutes (Gonzales de Galdeano et al., 1973). At the time of exchange the liver was removed from the perfusion vessel and perfusate allowed to drip from the liver into a collection beaker. Freshly oxygenated buffer at 37° was then poured into the vessel in batches, never allowing it to drain completely. After 150 ml fresh buffer had been perfused through the liver, the liver was returned to the perfusion vessel and experimentation continued.

The bile flow rates of the perfused livers were found to increase immediately in response to buffer exchanges even though the overall bile flow rates were found to decline in an exponential fashion. See Figure 12. Several possible explanations were proposed for these phasic responses which were encountered following the buffer exchanges. The most likely possibilities were 1) pH changes, 2) osmolarity changes, 3) oxygenation differences, 4) temperature changes, 5) production by the liver of metabolites or particular substances that feedback on certain hepatobiliary mechanisms, 6) decreasing levels of substrate, or 7) mechanical reasons.

After monitoring the pH, osmolarity, oxygen tension, and temperature before and after buffer exchange, no significant changes were

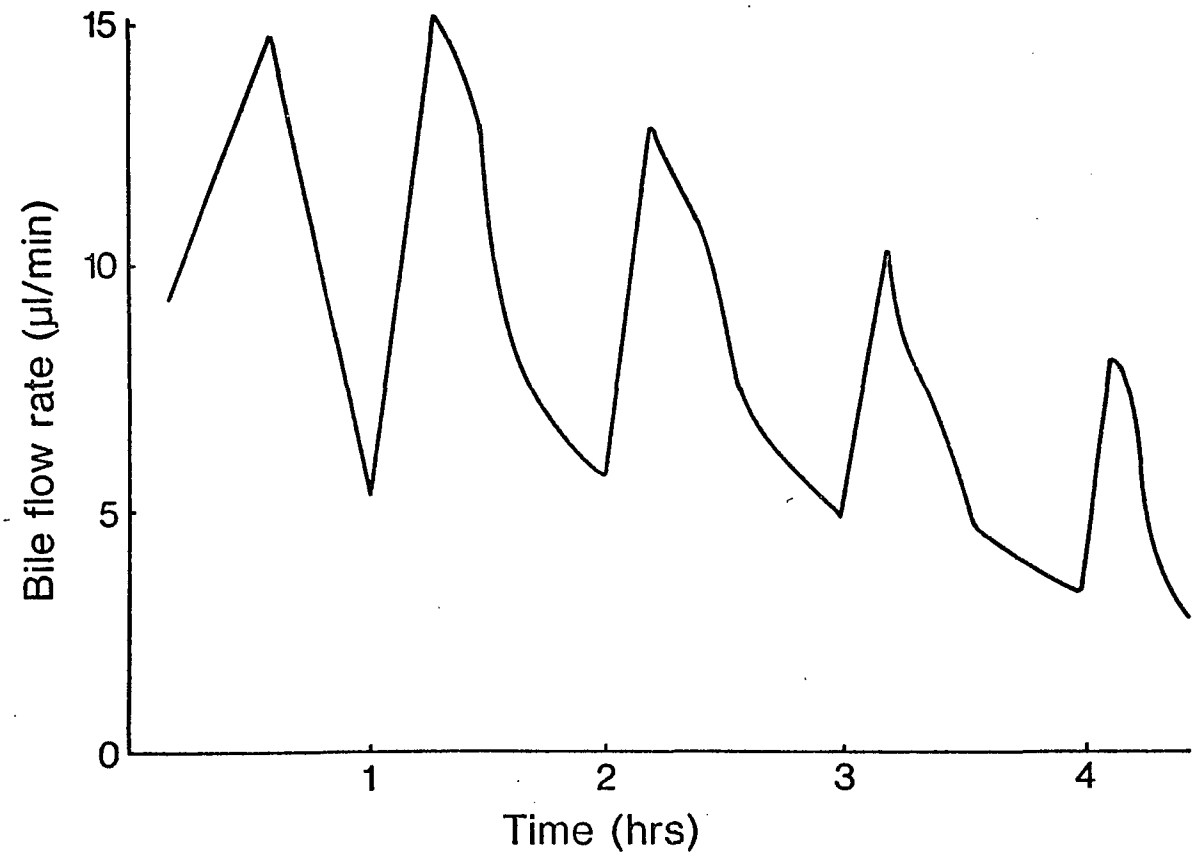


Figure 12. The effect of buffer exchange upon bile flow rates in the isolated perfused rat liver.

Bile flow rates (µl/min) were measured between buffer exchanges. At the end of each consecutive 60 minute perfusion period fresh pre-warmed, oxygenated buffer containing 5 mM pyruvate was exchanged for the used perfusate.

found (Table 1). As predicted it was found that changing the position of the liver relative to the bile duct cannula under certain conditions effected overall flow rates. For this reason a four way valve was included in the circuit in such a way that the liver then remained in the perfusion vessel during buffer exchanges in one predetermined and fixed position while new perfusate was sucked through one path of the valve into the system and the spent perfusate exited through the other valve path. However, even in the absence of mechanical obstructions, fluctuations in bile flow remained as dramatic in response to buffer exchanges as seen before. When 2 ml of a 5 mM solution of pyruvate was added at the end of a 60 minute perfusion period the bile flow was seen to increase significantly (Figure 13). In comparison however, administration of the same quantity of pyruvate solution at various time points in relation to the buffer exchanges affected the rate of bile flow to a lesser degree (Figure 14). One potential explanation of this phenomenon which is not mimicked by sodium chloride is that pyruvate itself as compared to lactate is more efficient in delivering the energy necessary for bile formation and that the fluctuations of bile flow observed following buffer exchange reflected the quantity of pyruvate present and rate of reduction to lactate, which is not as good a substrate for bile flow maintenance. A 10 mM pyruvate buffer was alternated at every other buffer exchange with a 10 mM lactate washout and perfusion buffer for the respective 60 minute perfusion period to test this hypothesis. If such a hypothesis were true, bile flow would be expected to be higher during the 60 minute pyruvate perfusion periods and lower during the 60

Table 1. Measurements of buffer pH, osmolarity, oxygen tension, and temperature at various time points.

Perfusion Time (min.)	pH	Osmolarity (mOsm)	Oxygen Tension (mm Hg)	Temperature (C°)
10	7.49	308	202.4	37
35[a]	7.36	302	194.1	37
60[a]	7.33	311	196.0	37
75[b]	7.41	308	192.4	37

a Measurements were performed on the initial perfusion buffer.
b Measurements at 75 minutes were performed after the original buffer had been exchanged at 65 minutes perfusion time.

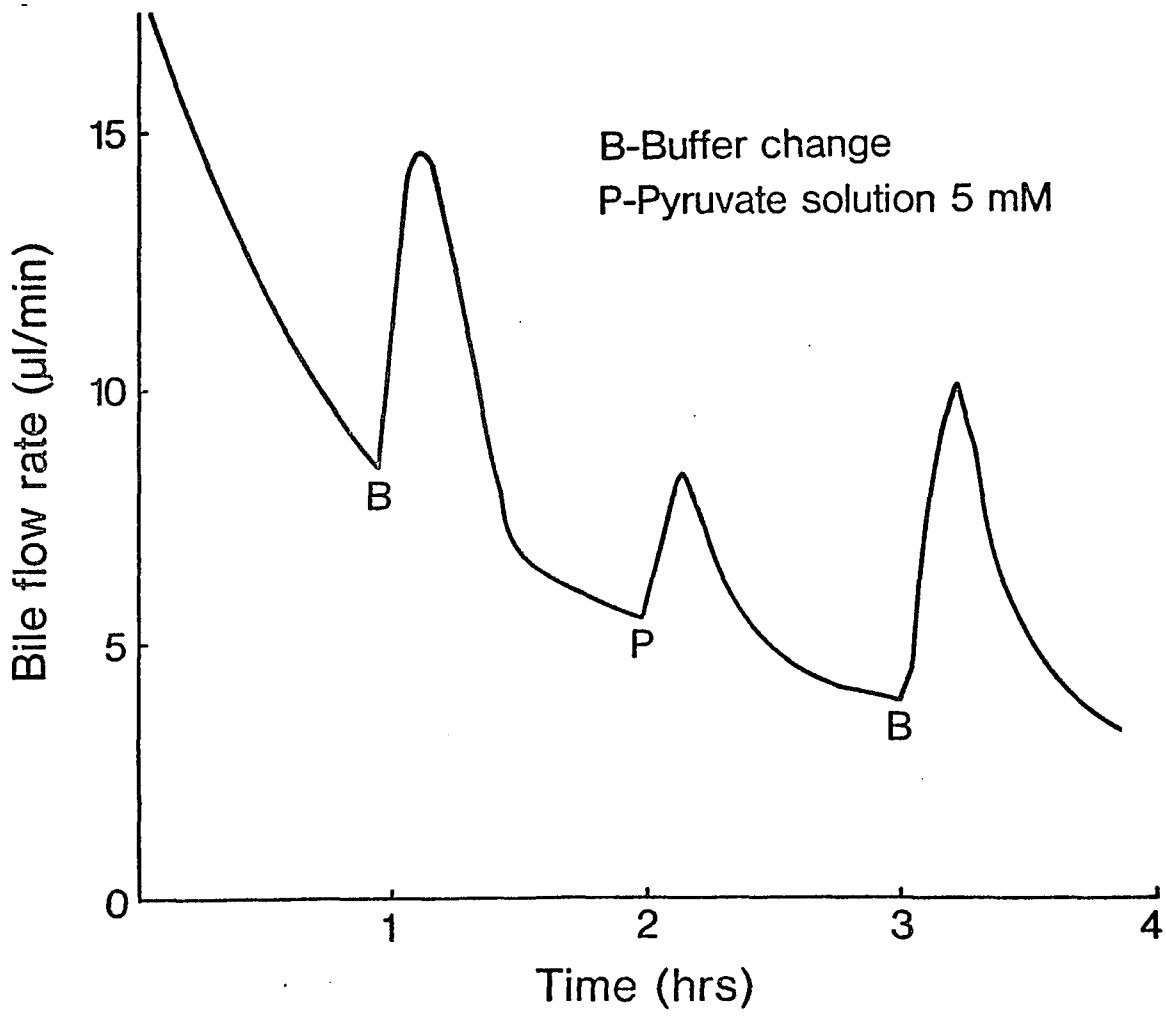


Figure 13. Effect of pyruvate administration upon bile flow in the isolated perfused rat liver.

A pyruvate solution, 2 ml 5 mM, was administered at the 2 hour time point instead of the routine buffer exchange.

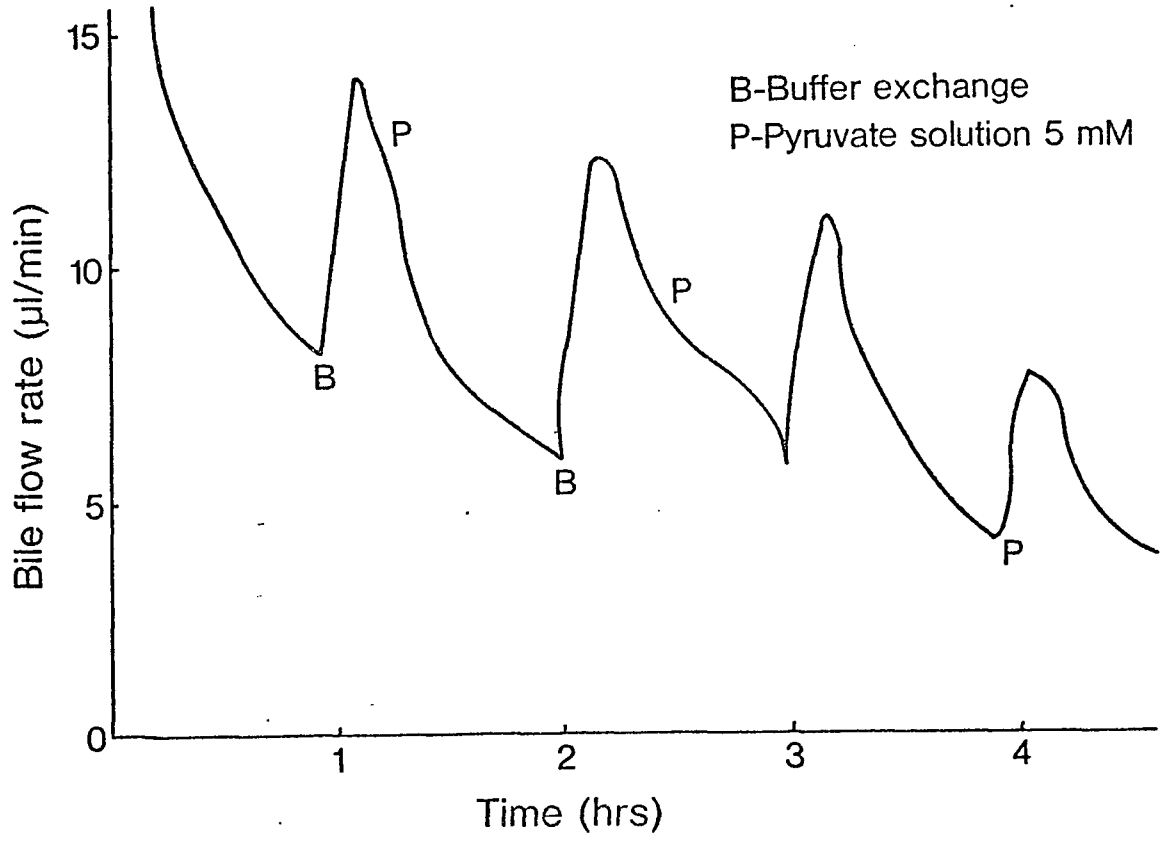


Figure 14. Effect of pyruvate administration at various time points in the isolated perfused rat liver.

Pyruvate solution, 2 ml 5 mM, was administered at various time points in relationship to the buffer exchanges. Bile flow was measured concurrent with pyruvate administration.

minute lactate periods. As seen in Figure 15 such a relationship was found to exist, demonstrating that pyruvate is a more efficient substrate for producing bile flow than lactate. In part, the fluctuations seen in bile flow rates following buffer exchanges could be attributed to a changing level of pyruvate substrate.

Since existing data concerning factors contributing to bile flow have shown that bile flow is primarily determined by 1) bile acid dependent secretion (Preisig, Cooper, and Wheeler, 1962; Dowling, Mack, and Picott, 1969), and 2) bile acid independent secretion, considered to be primarily due to sodium (Boyer and Klatskin, 1970), it seemed most logical to look at the effects produced by pyruvate and lactate on biliary bile acid and sodium secretion inorder to determine the cause for the discrepancy in bile flow rates between these two substrates. The sodium secretion rates observed during pyruvate perfusion periods were higher than during the lactate perfusion periods. However, no significant differences in total bile acid secretion rate were detected when comparing the two substrates (Figure 16). The sodium secretion rates could explain why pyruvate perfusion periods resulted in higher bile flow rates.

In any case, to maintain a constant substrate level it was decided to change the perfusion system in such a way that this would be possible. One obvious way to achieve this was the introduction of a dialysis circuit --essentially an artificial kidney-- into the perfusion flow scheme. If then on the dialysate side of such a membrane or hollow fiber dialysis device concentrations of substrate were maintained at

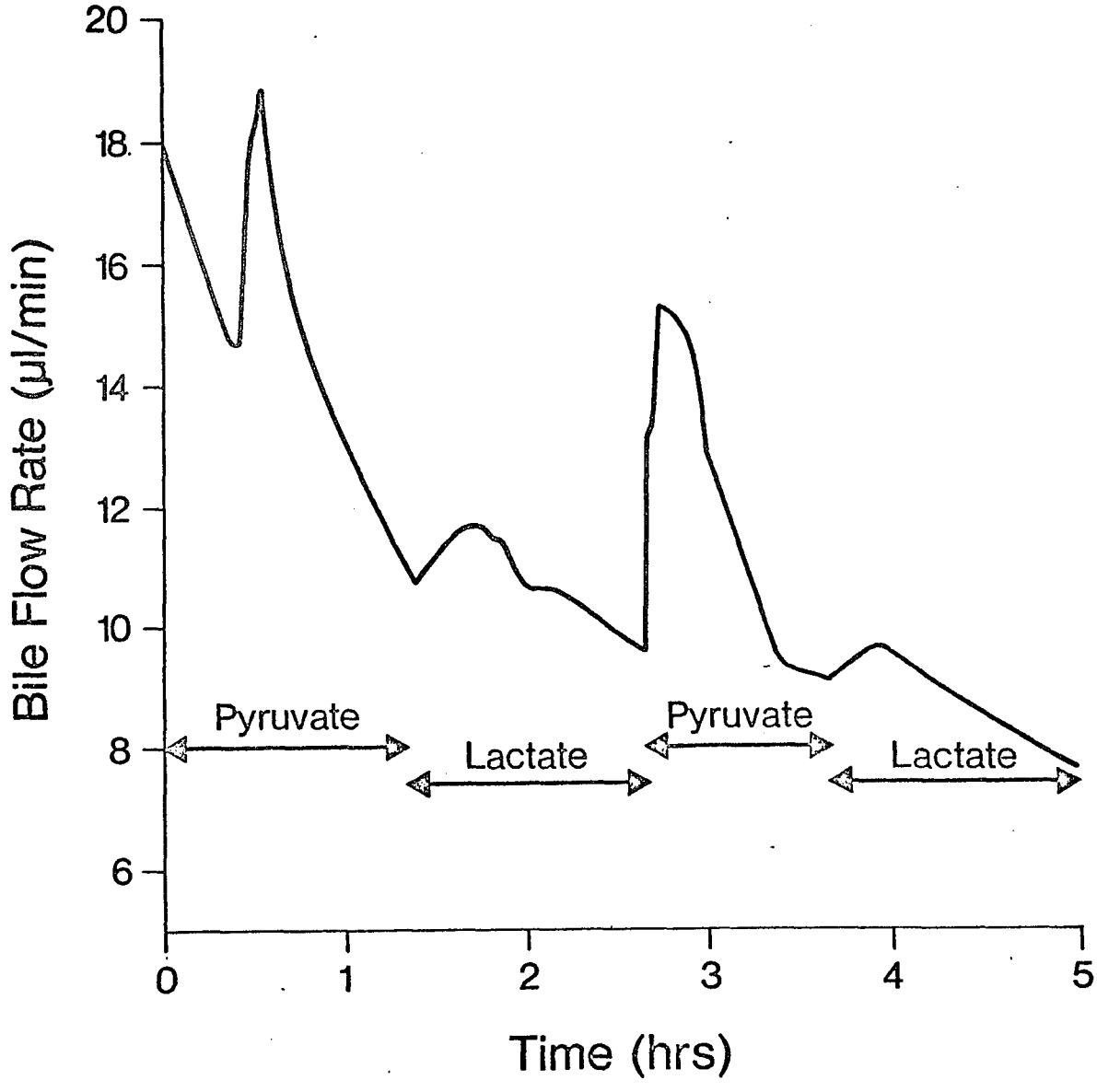


Figure 15. Changes in bile flow rate in response to alternating pyruvate (10 mM) and lactate (10 mM) perfusion buffers.

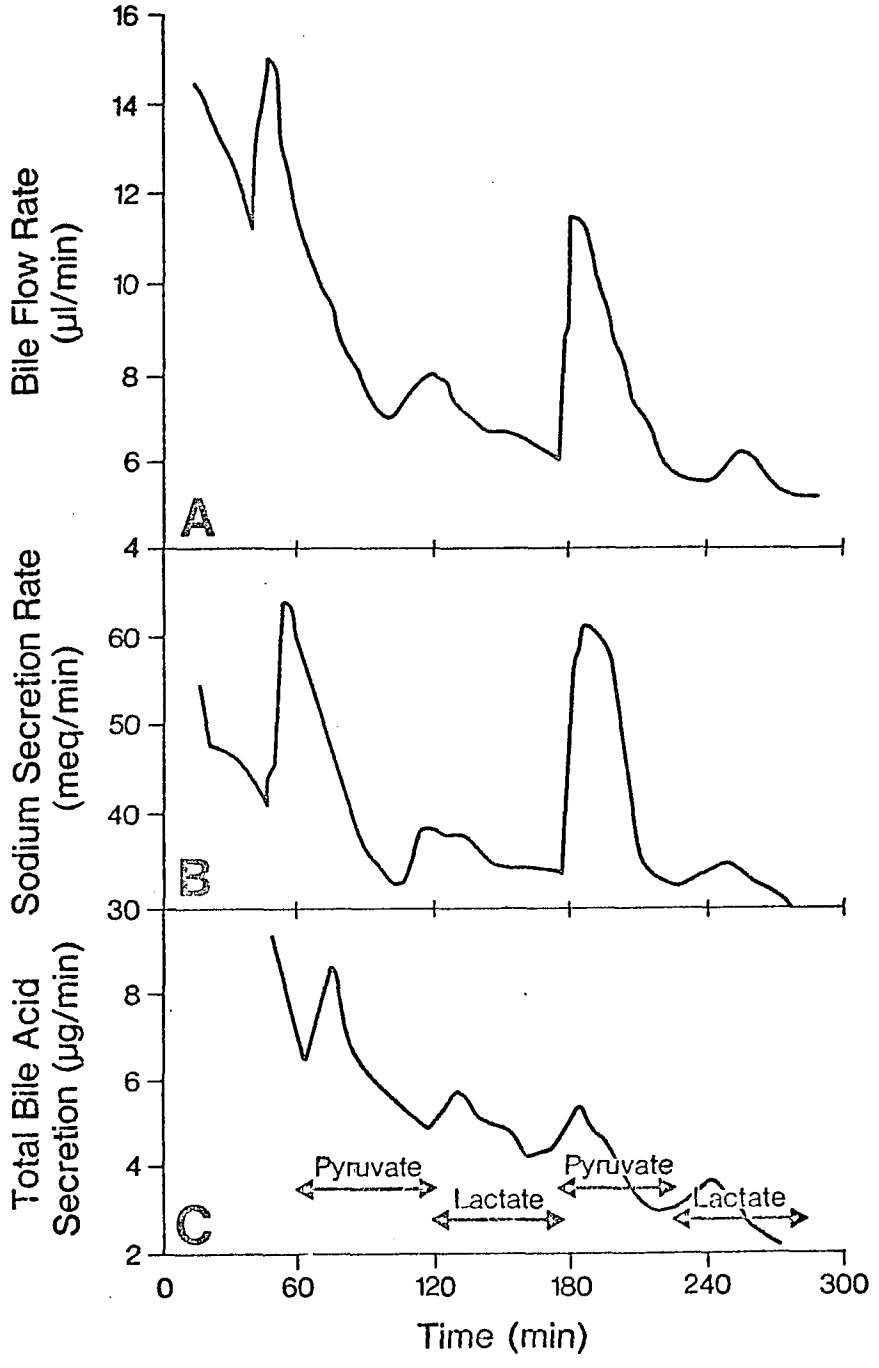


Figure 16. The effect of alternating pyruvate and lactate perfusion buffer upon A- bile flow rate, B- sodium secretion rate, and C- total bile acid secretion in the isolated perfused rat liver.

certain levels, these levels would reflect themselves on the perfusate side and would eliminate fluctuation of substrate supply to the organ. In addition, such a dialysis unit would tend to remove any molecular species below the membrane permeability cut-off (in this case 5000 M.W.) which is not present in the dialysate and is secreted by the liver. Although a constant level of substrate is attained, the metabolic state under which the liver is subjected is complex. When pyruvate is the substrate the equilibrium of the lactate dehydrogenase catalyzed reaction is strongly in favor of the reduced substrate. The first event after cellular uptake of the pyruvate is the reduction to lactate. This lactate then can reenter the extracellular space and thereby the perfusated. The consequence is a depletion of cellular reducing equivalents, a decrease of pyruvate and an increase of lactate. When the dialysis unit is incorporated in the perfusion circuit this lactate, not counterbalance by lactate on the dialysate side, would be diffusing out of the perfusate and would allow more pyruvate to flow in the opposite direction. The overall effect would be that an equilibrium between the two diffusion processes at the membrane and the lactate dehydrogenase reaction would be established under the maintenance of a constant intracellular pyruvate level and a constant drain on cellular reducing equivalents. This artificial physiological state would be characterized by an abnormally high oxidative state and can be experimentally contrasted with a high reductive state by utilizing lactate or lactate in the presence of ethanol as substrate for which a similar series of events apply. The ethanol enters the cell and is oxidized by lactate dehydrogenase

with formation of NADH and subsequent formation of acetate. The acetate can easily diffuse into the dialysate, whereas the NADH is retained within the cell ultimately leading to an abnormally high reductive state.

As anticipated, the addition of the dialysis unit to the perfusion system resulted in bile flow rates and bile acid secretion rates which no longer fluctuated in periodic phases. The bile flow rate and bile acid secretion rate dropped linearly during the first two hours and then stabilized in a linear fashion at a slower rate of decline during the remainder of the perfusion period as depicted in Figure 17. To insure that the dialysis unit was effectively producing a steady substrate concentration in the circulating buffer, lactate levels in the circulating perfusate were determined and monitored for 6 hours in experiments with 10 mM lactate. As is indicated in Figure 18 lactate levels after the initial 60 minutes were maintained stable between 4.0 and 4.8 mM. In a similar set of experiments using 10 mM pyruvate, stable levels in the perfusate were seen after one hour and were close to 2 mM. These stable levels of substrate in the perfusate of pyruvate livers are illustrated in Figure 19.

Before additional experiments could be carried out in more detail it was important to determine the viability of such a perfusion system. Therefore, perfusate levels of potassium, glutamic oxaloacetate transaminase (GOT) activity, and lactate dehydrogenase (LDH) activity, as well as vascular responsiveness were considered as parameters capable of indicating, at least in general, the physiological and

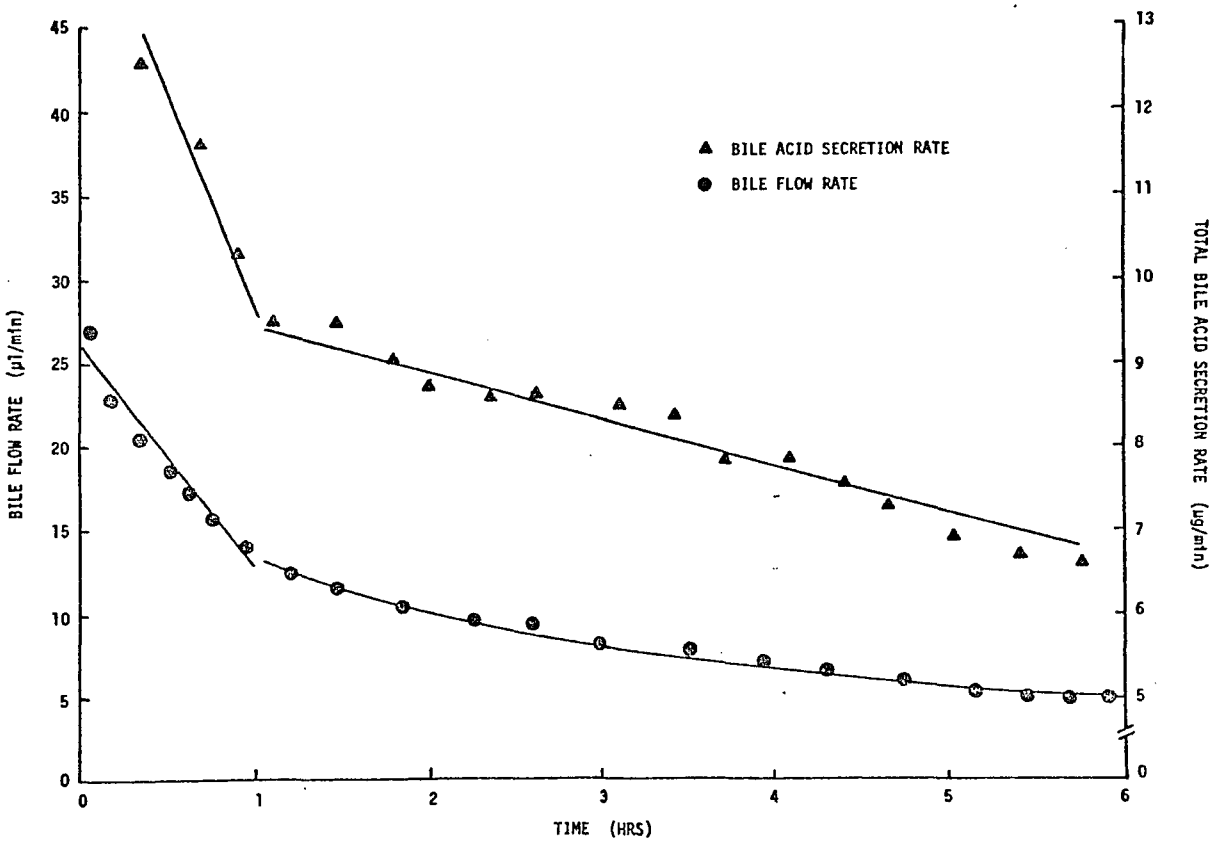


Figure 17. Bile flow rates and total bile acid secretion rates as affected by the addition of a dialysis unit to the perfusion system.

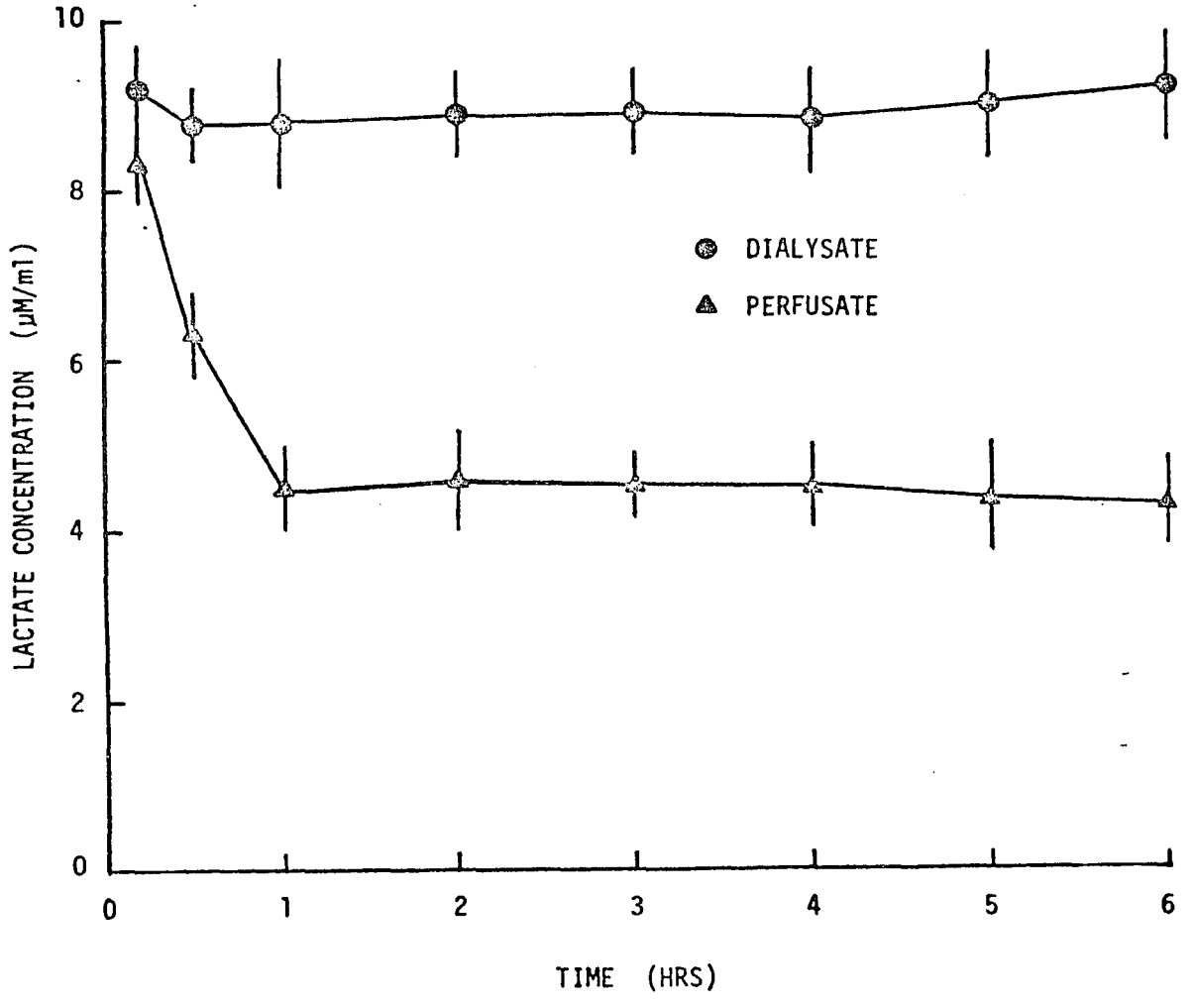


Figure 18. Lactate levels in circulating perfusate and dialysate for a set of rat liver perfusions including a dialysis unit.

Starting concentrations were 10 mM lactate in both perfusate and dialysate. Concentrations stay stable after one hour.

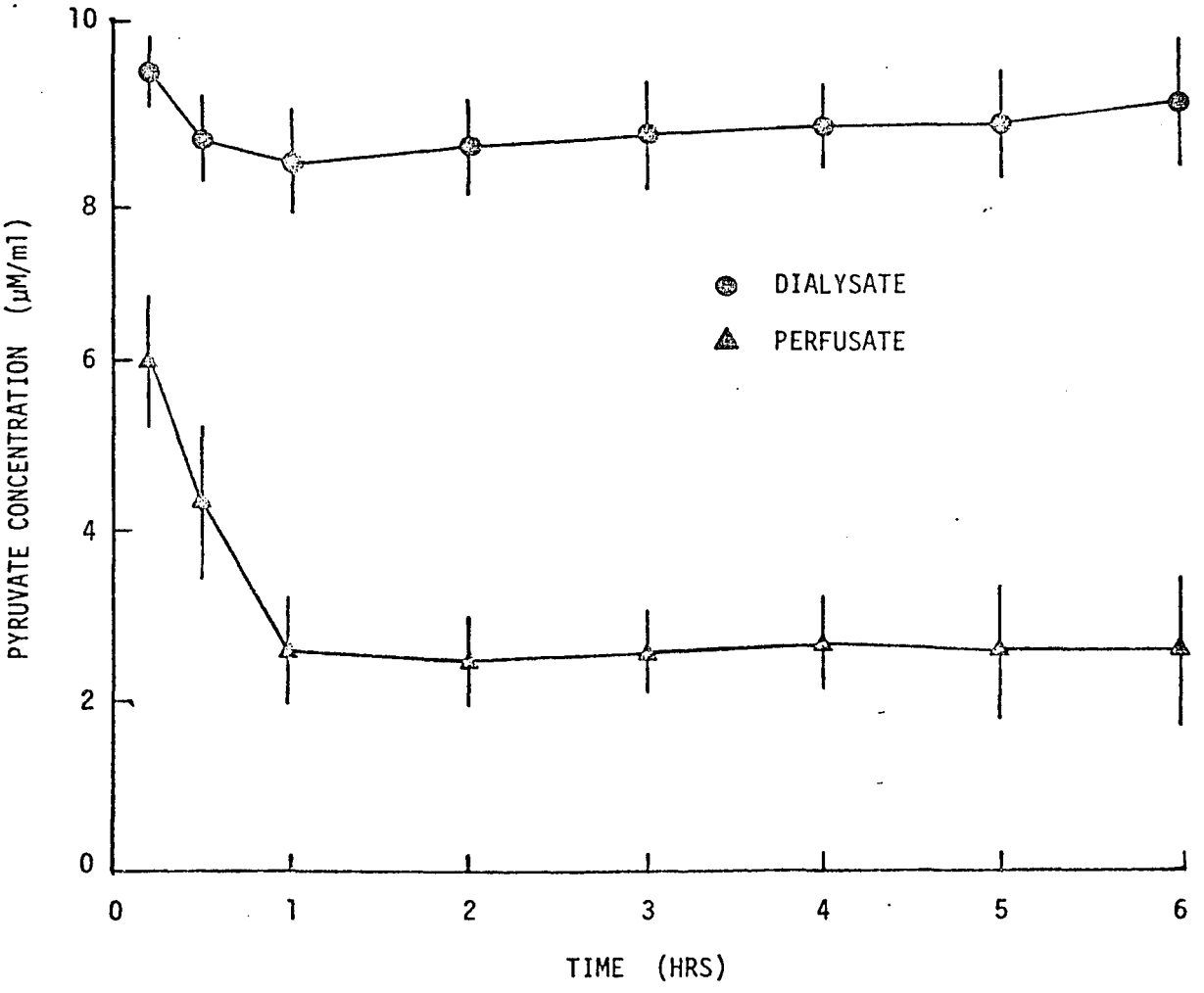


Figure 19. Pyruvate levels in circulating perfusate and dialysate for a set of rat liver perfusions including a dialysis unit.

Starting concentrations were 10 mM pyruvate in both perfusate and dialysate. Concentrations remain stable after one hour.

biochemical state of the perfused liver. In addition to testing the viability of this system, it was felt that these experiments would also allow a more complete characterization of the isolated perfused liver preparation.

The concentration of potassium in perfusate coming directly out of the liver was monitored such that small changes in liver potassium content could be detected throughout the perfusion. This was accomplished by cannulating the superior vena cava at the site of its exit from the liver and collecting samples at 15 to 60 minute intervals during perfusions. Figure 20 shows that during the first 30 minutes of perfusion the liver rapidly took up potassium to compensate for losses during the operation for removal and transfer to the perfusion circuit. The fact that the potassium concentration in the liver effluent remained essentially constant from this point on, showed that there was no subsequent leakage of potassium from the liver. As Bloxam (1971) has pointed out, if the potassium recovery represents the majority of potassium lost by the liver during the operation and transfer procedures, then the quantity of potassium taken up is a measure of that loss and is an indication of the degree of damage sustained. This reasoning is supported by their data showing that the initial potassium recovery is inversely related to the ATP content of the perfused livers at two and three hour time points. These potassium measurements therefore provided a sensitive and continuous measure of the condition of the liver which has meaning and is independent of the presence of different substrates. In my experiments a perfused liver was assumed intact

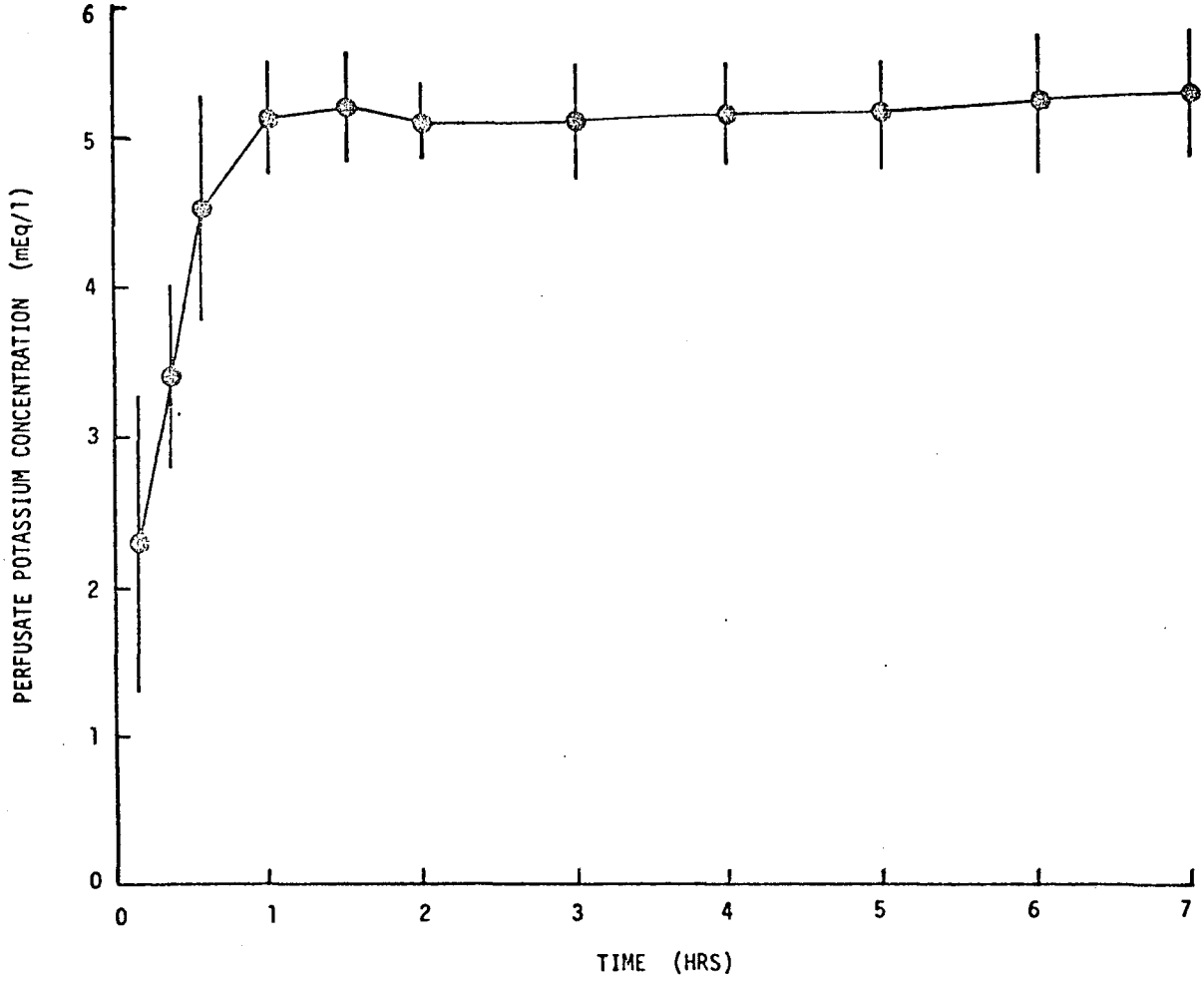


Figure 20. Change in perfusate potassium concentrations during perfusions.

The bars indicate the standard error (SE) for four liver perfusions in which pyruvate (10 mM) was the substrate.

if the initial potassium recovery time was less than 30 minutes and if there was no measurable loss of potassium during the remainder of the perfusion. In addition to these experiments, potassium measurements were routinely made in the bile samples collected during perfusion and the appearance of a significant rise of biliary potassium concentration in these samples was also taken as evidence for a deteriorating preparation.

Release of glutamic oxaloacetate transaminase (GOT) or glutamate pyruvate transaminase (GPT) from the liver has been shown to be a sensitive measure of liver cell damage (Hess, 1963). The activity of GOT in the perfusion medium slowly increased over time, more so after four hours, indicating a tolerable level of organ deterioration (Figure 21). The total release of GOT from livers perfused at least 8 hours was 7-12% of total activity.

Since lactate dehydrogenase (LDH) is of clinical usefullness (Jordon and White, 1967) in diagnosis of liver disease this enzyme activity was also intermittantly checked in the perfusion medium. Figure 22 illustrates the change in LDH levels during 8 hour perfusions. The LDH activity appears to increase linearly up to 6 or 7 hours after which the rate of enzyme release was found to accelerate moderately. The liver is known to synthesize and secrete LDH (Miller and John, 1970) even in the absence of cell damage. Therefore, it was not surprising to find an increasing enzyme activity over time. However, to test the premise that functional damage would appear as an increased rate of LDH activity above the normal synthetic rate, 50 µl

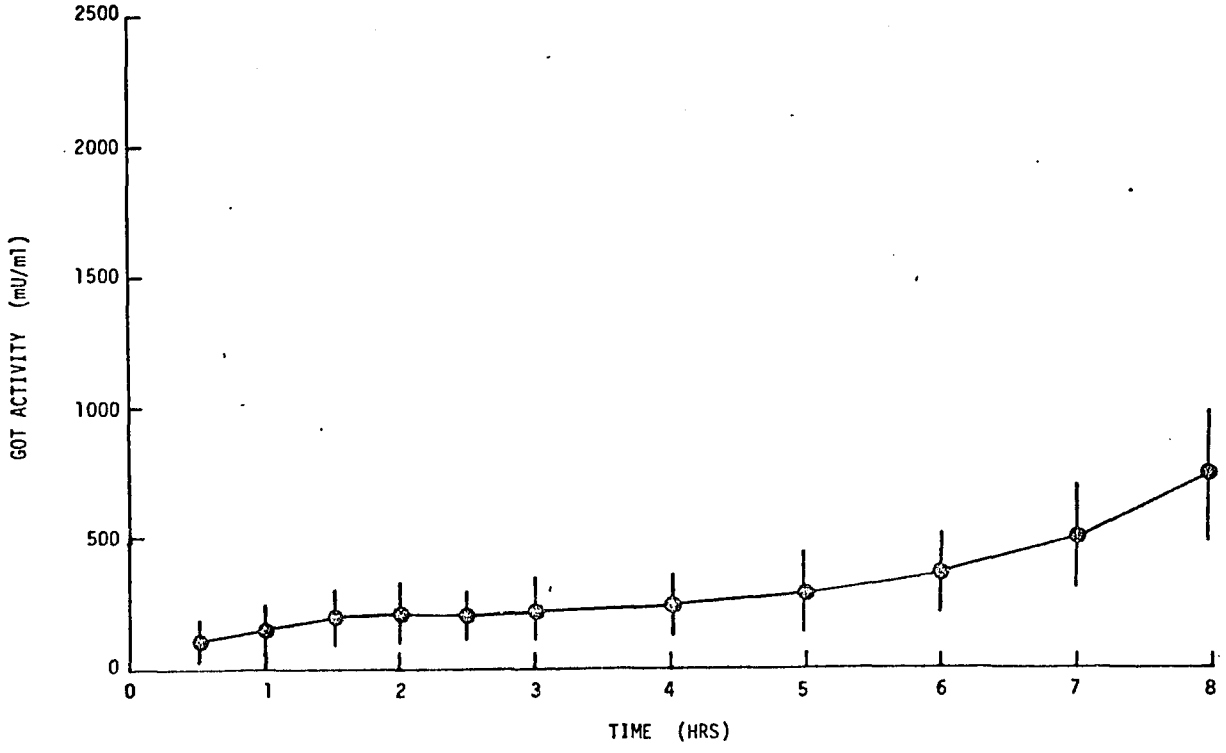


Figure 21. Appearance of glutamate oxaloacetate transaminase (GOT) activity in the circulating medium during perfusion of isolated livers over an hour time period.

The bars indicate the standard error (SE) for four liver perfusions in which pyruvate (10 mM) was the substrate.

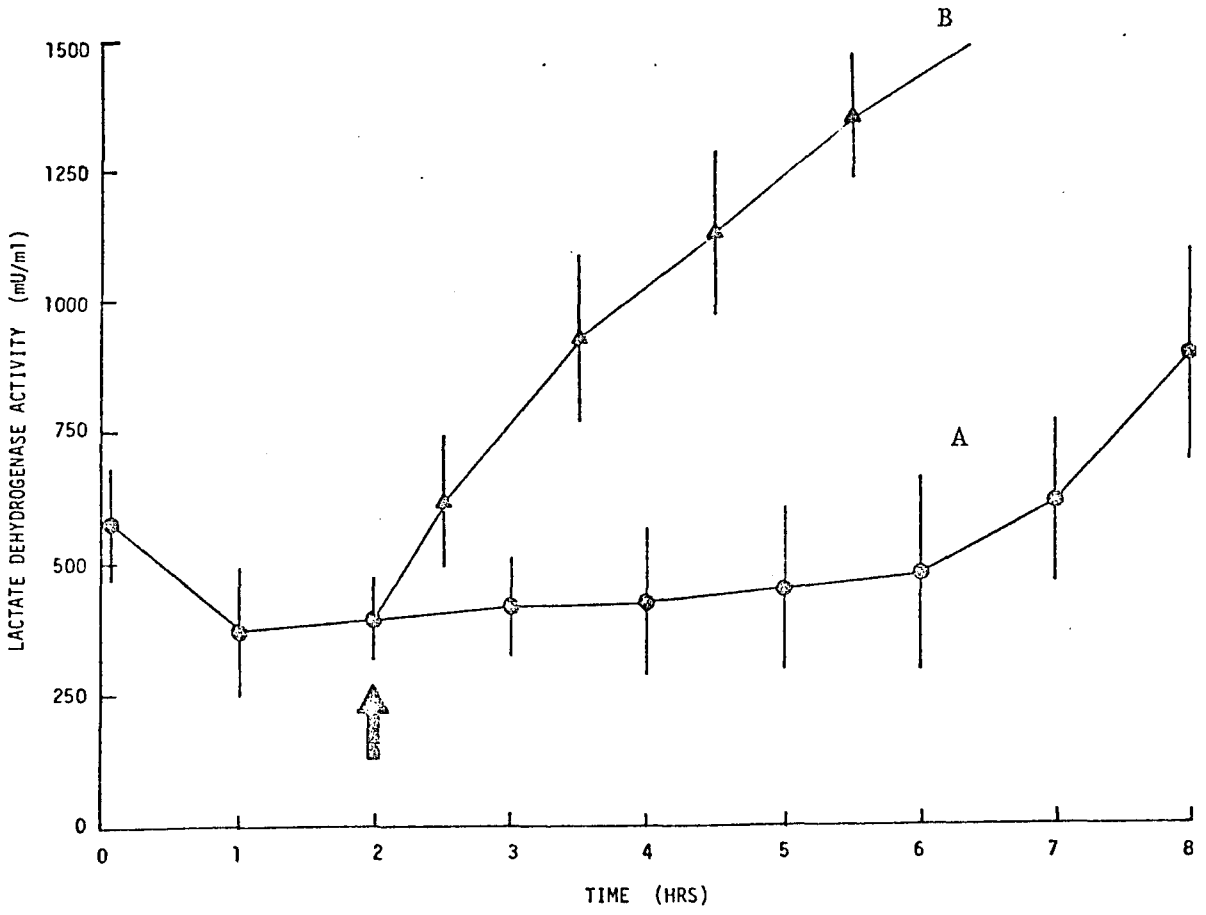


Figure 22. Appearance of lactate dehydrogenase (LDH) activity in the circulating medium of isolated livers during control perfusions (A) and perfusions administered carbon tetrachloride (B).

At the arrow 50 µl carbon tetrachloride was administered to 3 perfused livers and the LDH compared to 3 control perfused livers. The bars indicate the standard error (SE) for 3 control (A) and 3 carbon tetrachloride treated perfused livers.

of carbon tetrachloride was added to the perfusion medium. Carbon tetrachloride caused the immediate and continuous release of LDH activity at a rate 3 to 4 times the control perfusions. The data seen in Figure 22 indicate that the control perfusions were functioning within a normal range up to 6 or 7 hours but also showed that beyond this time definite degradation was taking place. For this reason the duration of most of the perfusions were limited to 6 hours, to insure the validity of a viable system when evaluating other parameters.

Flow through the portal system shows a high adaptability (Brauer, 1963) and in certain animal species the portal system operates as a constant flow system and the increased resistance is manifested as a rise of portal vein pressure (Greenway, Lawson, and Mellander, 1967). A constant flow was used in all experiments and it appeared that for the parameters checked, both constant flow and constant perfusion pressure techniques are equivalent (Miller et al., 1951), although a constant pressure system was never tested. Previous empiracle experience from this laboratory has suggested that portal vein pressure is a good indicator of general liver condition. For this reason, a monitoring device was used as described in the Methods and Materials. Other investigators have shown that the portal pressure rises significantly over perfusion time (Kestens, 1964; Bartosek, Guaintani, and Garattini, 1973). Figure 23 demonstrates that in contrast, when utilizing the dialysis system, pressure is maintained relatively constant over a 6 hour period. Although the portal pressure remained stable after the initial drop, continuous monitoring led to an observation of

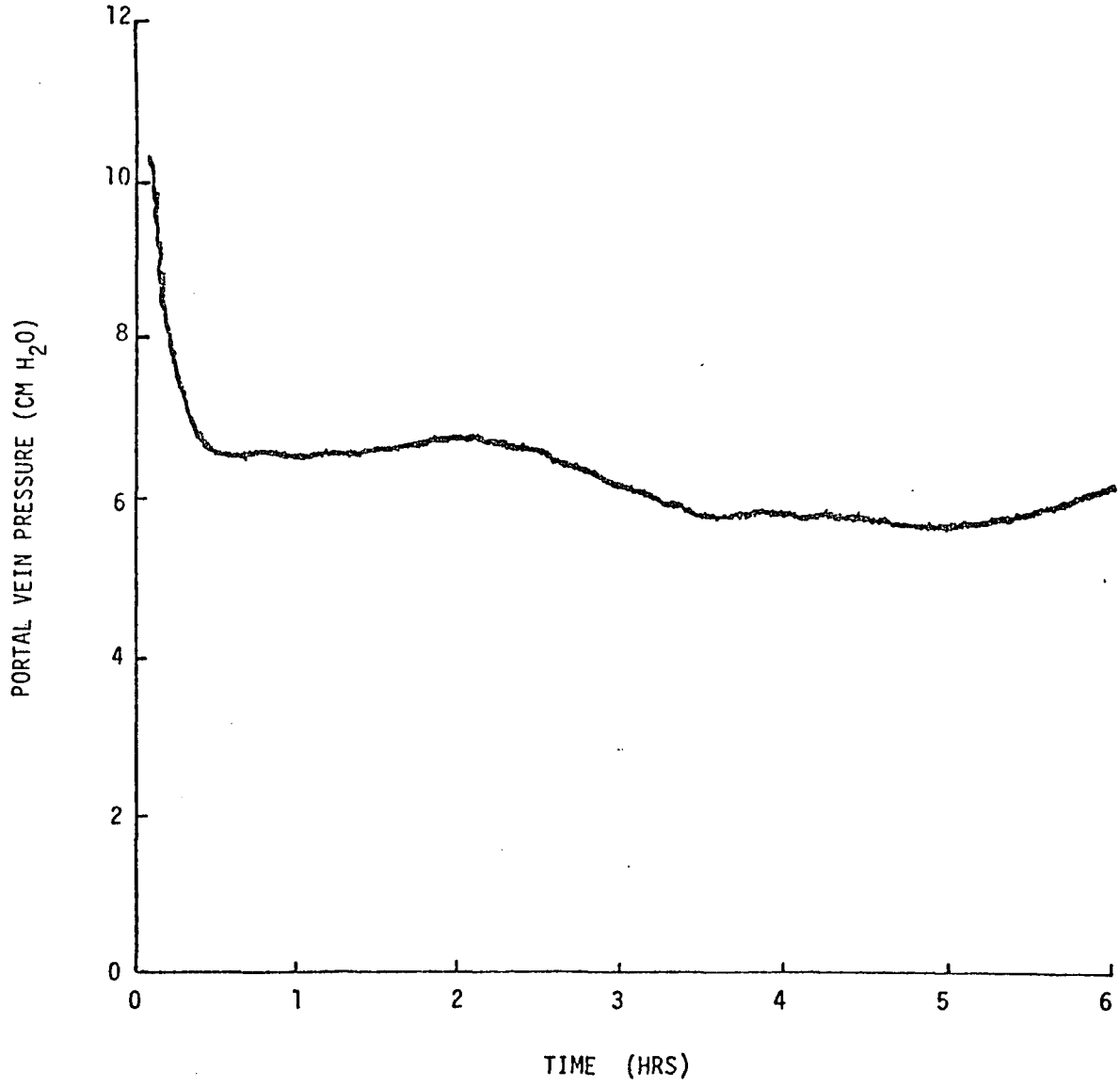


Figure 23. Typical changes observed in portal vein pressure over a 6 hour perfusion period.

additional interest. During various perfusions, cyclic fluctuations in pressure were recorded (Figure 24). One possible explanation was that the liver was in effect trying to regulate flow by changing the internal resistance. Since not every perfused liver elicited such a response, plus the fact that isolated livers are not innervated, led to the postulation that the isolated perfused livers were demonstrating metabolic regulation of flow (Rothe and Friedman, 1971) coupled to lactate formation.

A possible reason every isolated liver did not respond in a similar manner could be interpreted as a lack of synchronization of the pressure changes throughout the organ which would be necessary to observe such a response. Various procedures were used to try to synchronize the livers in respect to this phenomenon such as adding a bolus of lactate or ethanol, producing a period of severe anoxia by discontinuing oxygenation for a period of time or constriction with pulsed norepinephrine at a concentration of 1 μg/ml. Although the addition of 5 mM pyruvate and 0.1 mM ethanol followed by norepinephrine initiated the response more often than any other combination of procedures (Figure 25, panel C), the pressure fluctuation pattern could not always be reproduced (Figure 25, panel A and B). To test the hypothesis of chemical regulation of flow in the perfused livers a continuous assay system for lactic acid was set up to detect rhythmic changes in lactic acid effluent concentrations as described in the Materials and Methods section. A number of experiments were performed to determine if there was a relationship between pressure changes and lactate levels in the

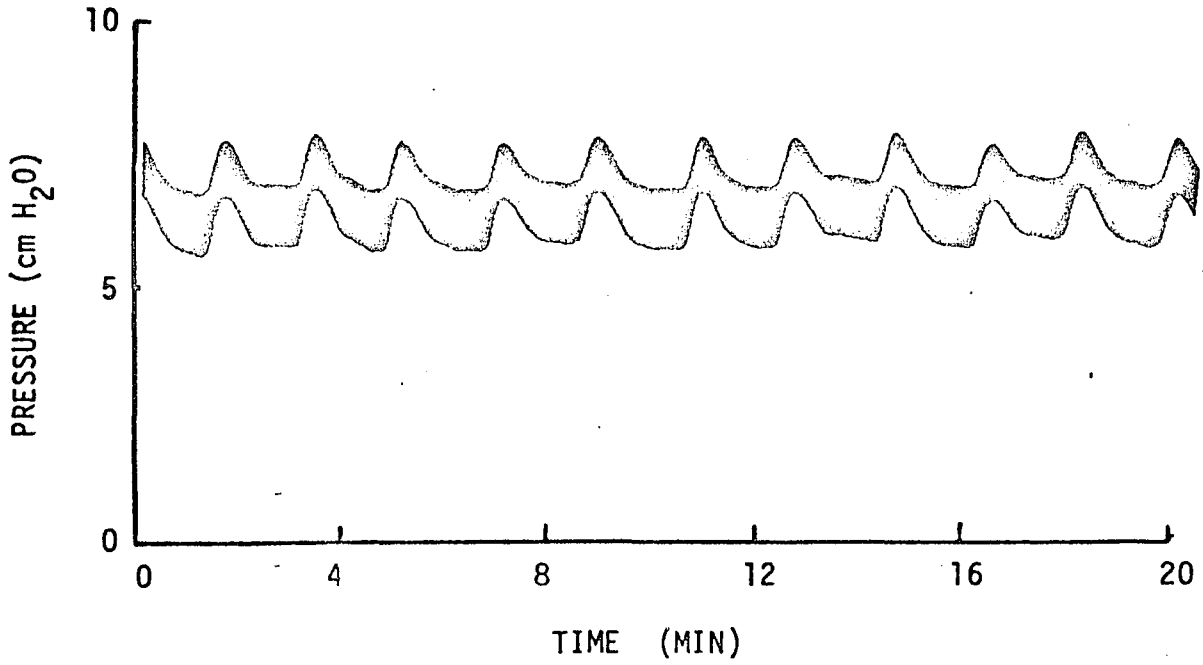


Figure 24. Pressure fluctuations observed periodically during normal liver perfusions.

These cyclic fluctuations in portal vein pressures were usually changes of 1-3 cm H_2O over a 2 to 3 minute time span.

Figure 25. Vascular responsiveness of the isolated perfused liver.

A. Effect of lactate upon portal vein tension.
B. Maximal constriction produced by norepinephrine (1 µg/ml) with no resulting pressure fluctuations.
C. Initiation of pressure fluctuation in response to administered norepinephrine (1 µg/ml) in the presence of 5 mM pyruvate. Pressure fluctuations were abolished by buffer washout.
D. Initiation of pressure fluctuation in response to administered norepinephrine (1 µg/ml) in the presence of 5 mM pyruvate. Pressure fluctuations were abolished by the administration of the beta-agonist, isoxuprine hydrochloride - 25 µg/ml.

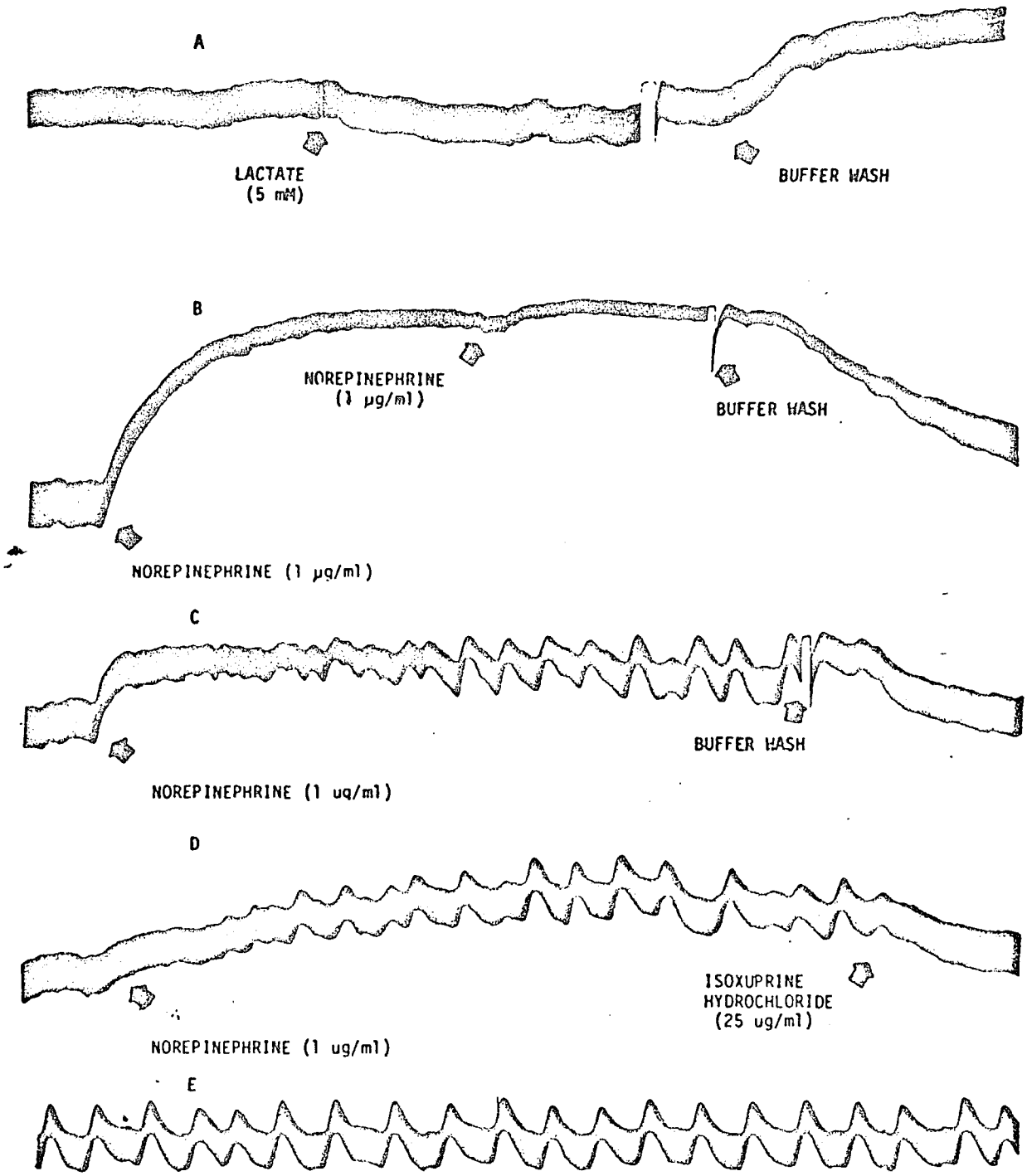


Figure 25. Vascular responsiveness of the isolated perfused liver.

perfusate immediately exiting the livers. Although a continuous increase in lactate concentration was observed, no rhythmic changes could be detected (Figure 26). Several possible reasons for the lack of expected response include 1) the lactate assay was not sensitive enough to detect the small lactate concentration changes, 2) although significant changes of lactate occur at the cellular level, the washout and mixing in the interstitium and sinus passages might dampen the changes, and 3) no relationship between pressure fluctuation and lactate production exists. Although the rhythmic changes were only observed under specific conditions, in all experiments differences in portal tension were seen in response to the various substrates used. Lactate (5 mM) produced a lower back pressure which increased when the lactate was washed out with perfusate containing no substrate (Figure 25, panel A). The same response was seen when changing from lactate to a 5 mM pyruvate buffer, suggesting that lactate can act as a vasodilator in the isolated liver. The norepinephrine induced pressure fluctuations could be explained by a maximum constriction (Figure 25, panel B and C) producing anoxic tissue throughout the perfused organ at the same time, which then would cause an increase in lactic acid formation. The build-up of lactic acid in synchrony then affects a short but significant vasodilitation until the increased flow washes out the higher lactate levels followed by a return of the norepinephrine effect. A similar overriding effect by anoxia or lactate of the high levels of norepinephrine effect may be produced as observed in cardiovascular shock (Haddy and Scott, 1968). It should be pointed out that although

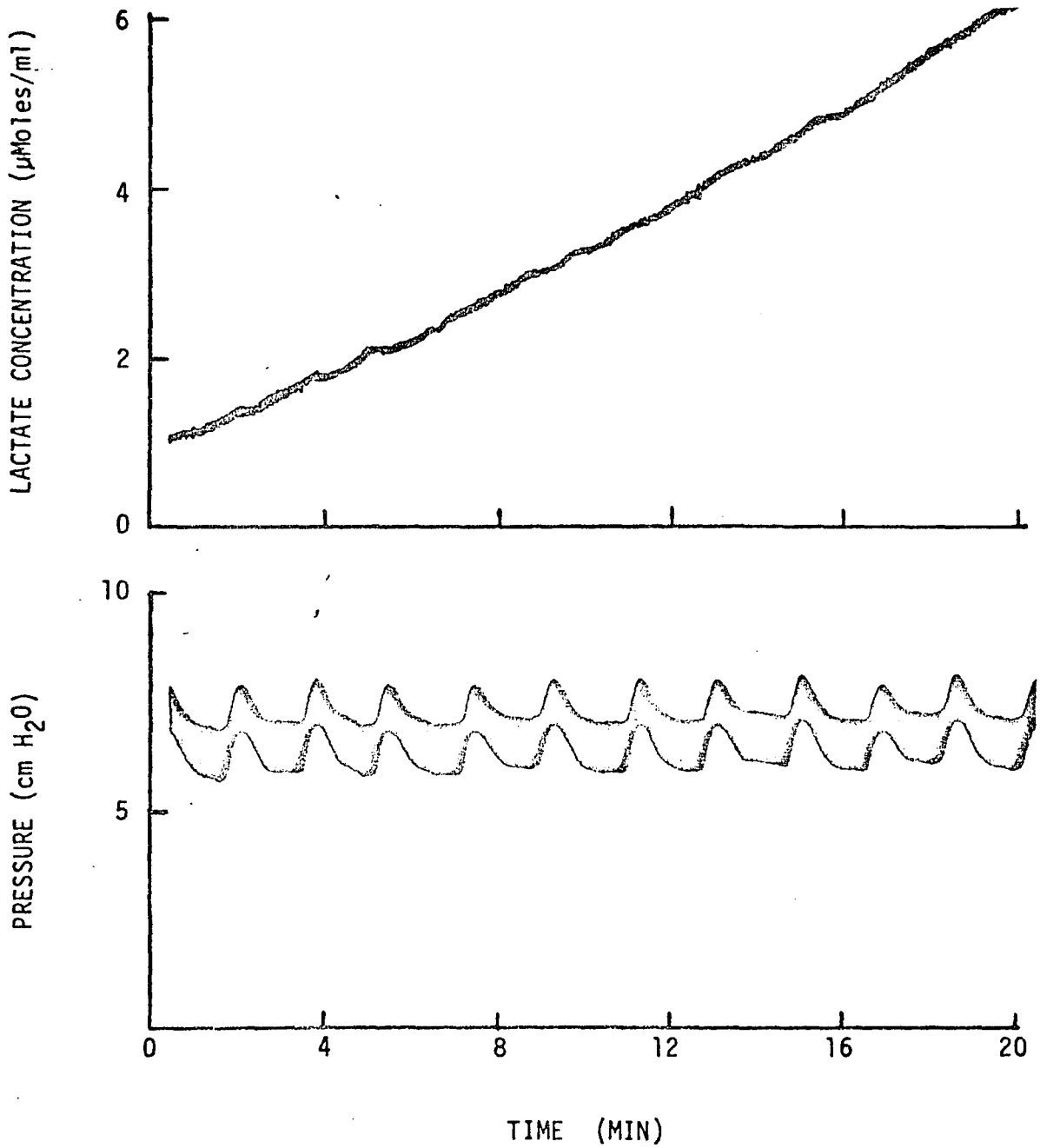


Figure 26. Lactate determination in direct effluent from the isolated perfused rat liver measured concurrently with the fluctuations in portal vein pressure.

the rhythmic pressure changes can sometimes be elicited by norepinephrine, the phenomenon as it was initially detected has been observed to occur spontaneously, implicating that the norepinephrine is only one possible stimulus. The addition of the beta receptor agonist, isoxuprine hydrochloride, during a period of pressure fluctuation demonstrated that the pressure changes are purely vascular and not derived from a mechanical origin (Figure 25, panel D). That both washout and isoxuprine hydrochloride abolished the pressure changes, further suggests that the response is due to a synchronization of several processes. Irrespective of the final interpretation, it has been clearly demonstrated that vascular receptors are present and responsive over 6 hours in the isolated perfused liver and this is thought to be another criterion for indicating the viability of the in vitro system. Other parameters which serve as function tests, such as general color and appearance, hepatic edema, bile flow and oxygen uptake, have been measured before and found to be equal or superior to values cited in the literature (Fisher and Kerly, 1964; Mayers and Felts, 1966).

An isolated liver perfusion system yielding relatively stable rates of bile flow and bile acid synthesis and secretion for 6 hours as has been described above, would be extremely useful in the evaluation of potential influencing factors which might have a direct effect on bile flow and bile acid synthesis and secretion.

The effects of dietary fats (McGovern and Quackenbush, 1973), alcohol (Lefevre et al., 1972), and glucose (Uchida et al., 1975) on bile acid parameters have been studied utilizing in vivo systems.

However, several uncertainties arise when using in vivo systems to study such effects. For example, the published results of Uchida et al. (1975) on bile acid metabolism after administration of a bolus of glucose to rats might possibly have been due to a secondary effect upon gut absorption of bile acids or due to a stimulation of insulin output followed by its possible effects on bile acid metabolism as well as its known effect upon bile flow (Jones and Brooks, 1967). Using the isolated perfusion system described herein, it is possible to eliminate secondary influencing factors and precisely control the experimental conditions, thus removing some of the hormonal and metabolic uncertainties encountered in in vivo systems.

Gustafsson and Norman (1969b) and Heaton (1972) have shown that positive correlation exists between the quantity of ingested refined carbohydrates and the incidence of gallstone formation. Since it is also generally assumed that changes in the liver metabolism of bile acids, cholesterol, and phospholipids initiate the formation of cholesterol gallstones (Swell, Gregory, and Vlahcevic, 1974), it became apparent that a series of long term liver perfusions should be initiated to determine the effect of substrates directly on the liver, and how this might effect changes in the hepato-biliary processes of synthesis and secretion of bile acids as well as bile flow. These experiments would hopefully advance the understanding of the mechanisms involved in altering changes in cholesterol and bile acid metabolism as produced by different oxidative states.

Since previous studies in this thesis have shown bile flow rates to be affected by the particular substrate used when alternating buffer exchanges between lactate and pyruvate, it was of interest to see whether similar results could also be achieved utilizing the long term perfusion system with the dialysis unit. As illustrated in Figure 27 the bile flow rates during a 6 hour period for 5 lactate and 5 pyruvate liver perfusions are relatively stable in decline. As expected the overall bile flow rates for lactate perfusions were found to be lower than pyruvate perfusions. Pyruvate and dextrose perfused livers were found not to be significantly different from each other, but both had significantly higher flow rates than the lactate perfused livers (Figure 27 B). In comparison, all 5 "no substrate" perfused livers were found to have greatly reduced flow rates beyond the two hour period with two of the liver preparations having no flow at the four hour mark (Figure 27 C). These results further substantiate the view that bile flow is dependent upon metabolic processes rather than mechanical processes such as perfusion pressure or flow rate (Garf, Korn, and Peterlik, 1973; Schmucker and Curtis, 1974).

As seen in previous experiments utilizing buffer exchanges, the total bile acids synthesized and secreted into the bile by each long term perfusion were determined. Since the secondary bile acids, lithocholic acid and deoxycholate, rapidly declined with time and after the first hour of perfusion were found to be absent, all bile acids analyzed in the bile samples after the first hour period were considered newly synthesized and secreted. Although no corresponding changes

Figure 27. Bile flow rates of isolated perfused rat livers as effected by the particular substrate perfused.

Panel A- Pyruvate and Lactate- Pyruvate livers had significantly higher ($P < 0.01$) bile flow rates at all time points except at 3 and 4 hours.
Panel B- Dextrose and Lactate- Dextrose livers, as did pyruvate livers, also had a significantly higher ($P < 0.01$) bile flow rates compared to the lactate livers at all time points except at one hour.
Note: bile flow rates for puruvate and dextrose livers were not significantly different from each other at any time point.
Panel C- Pyruvate and No substrate- The deterioation of bile flow for the no substrate livers demonstrates the necessity for adding substrate to maintain bile flow.

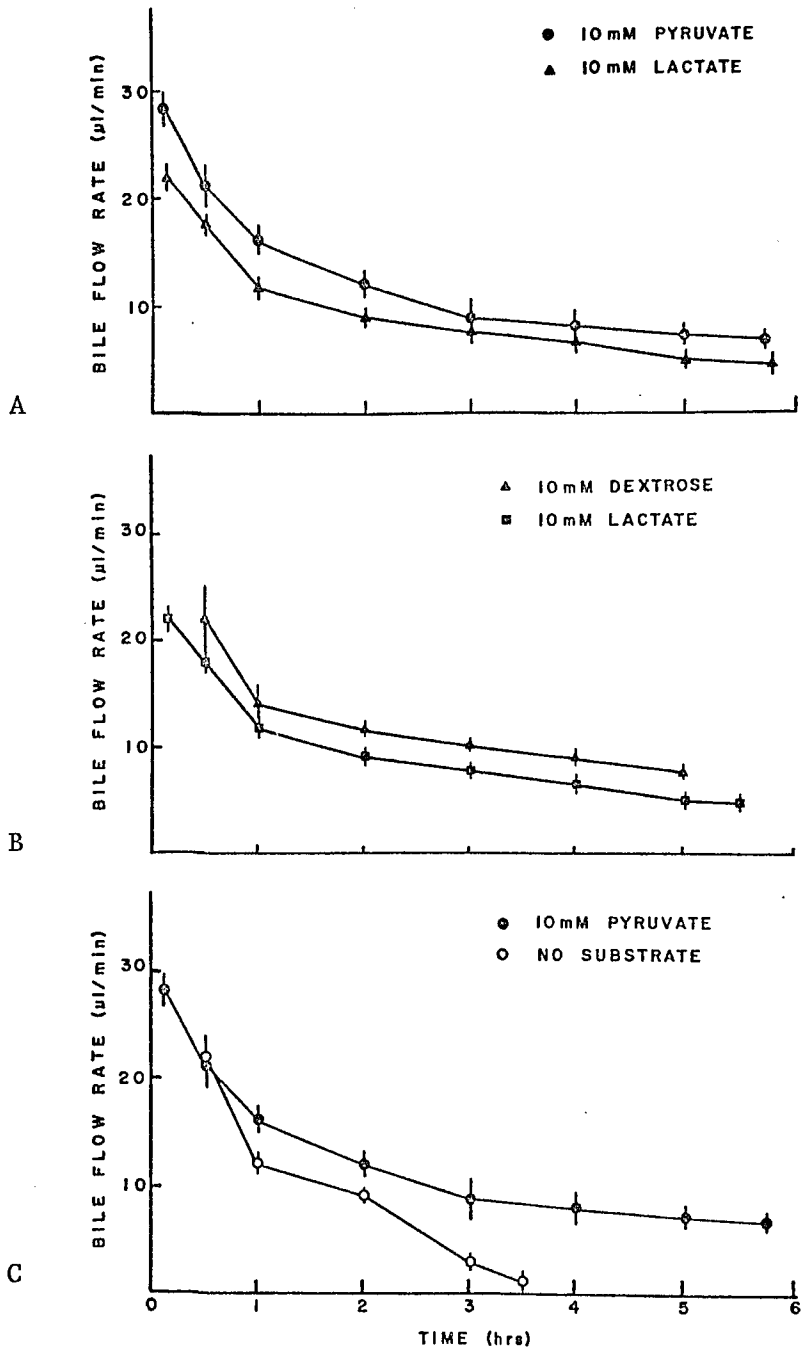


Figure 27. Bile flow rates of isolated perfused rat livers as effected by the particular substrate perfused.

in total bile acids synthesized and secreted were found with the changes observed for the bile flow rates over the one hour periods between buffer exchanges in the initial experiments reported (Figure 16 C), there was anticipation of finding alterations in total bile acids synthesized when comparing the different substrates over six hour perfusion times. However, after enzymatic analysis it was found that the total bile acid synthesis and secretion by these livers not to be significantly different from each other for all three substrates tested (Figure 28). It is important to remember that the normal enterohepatic circulation of bile acids is interrupted in this system, such that the rate limiting enzyme, cholesterol 7α-hydroxylase, which is usually responsive to bile acid feedback is uninhibited and concidered to be at maximal activity (Danielsson, Einarsson, and Johansson, 1967; Johansson, 1970). Therefore, the lack of effect upon total bile acid synthesis and secretion by the substrates in this system would only indicate that the substrates do not influence the rate limiting enzyme (s) or that all groups of perfusions were merely converting cholesterol into bile acids at the same maximal rate. In contrast, the perfusion experiments with no exogenous substrate in the buffer demonstrated that the substrates are necessary for the hepatobiliary process of bile acid synthesis and secretion providing further support for the concept that after the initial hour of perfusion only newly synthesized bile acids are secreted and that the precursor pool of cholesterol has to be replenished by synthesis.

Figure 28. Biliary bile acid secretion rates of the isolated perfused rat livers as effected by the particular substrate perfused.

Panel A- Pyruvate and Lactate- Pyruvate livers were found not to be significantly different from lactate livers except at time points 0.5 and 3 hours. Note: Dextrose livers were also found not to be significantly different form either the pyruvate or lactate livers.
Panel B- Dextrose livers, as well as pyruvate and lactate livers, were found to have significantly higher biliary bile acid secretion rates at all time points beyond 2 hours.

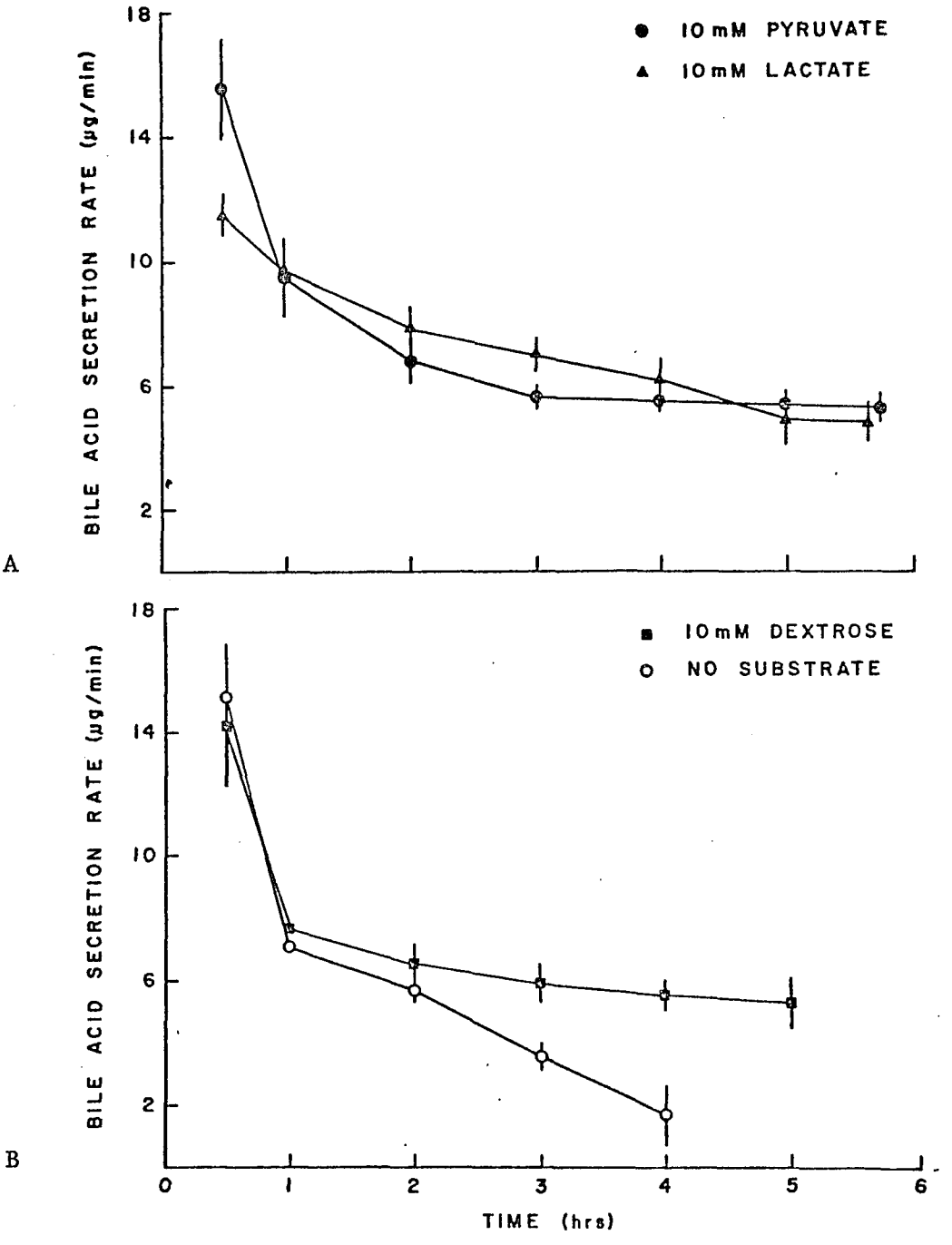


Figure 28. Biliary bile acid secretion rates of the isolated perfused rat livers as effected by the particular substrate perfused.

The results then indicate that the differences observed in bile flow rates in response to the substrates would have to be due to the bile acid independent processes. As expected, the pyruvate perfused livers secreted a significantly higher quantity of sodium into the bile when compared to livers perfused with lactate (Figure 29). This increase could easily explain why pyruvate livers consistantly had higher bile flow rates. The biliary sodium secretion for dextrose perfused livers, however, did not correlate with the differences in bile flow rates (Figure 29 B). It was expected that the dextrose livers would have a sodium secretion rate comparable to that of the pyruvate perfused livers since their bile flow rates were not significantly different from each other. The only explanation for this discrepancy is that the dextrose (10 mM) saturating the perfusate, ended up being secreted into the biliary tree thus producing an osmotic gradient and increasing the bile flow rates. Even though glucose levels were not measured in bile, support for this explanation can be found in the work of Jones, Geist, and Hall (1970) who demonstrated that the biliary levels of substances such as 2-deoxyglucose and amino acids do increase as their plasma levels are raised.

Although there was no significant change in total bile acids secreted in response to these substrates, the individual bile acid profiles were found to be influenced by the substrate present during perfusion. Before going into a detailed analysis of these changes some basic facts on the separation of bile acids should be mentioned. Figure 30 illustrates the gas liquid chromatographic (GLC) separation of

Figure 29. Biliary sodium secretion rates of the isolated perfused rat livers as effected by the particular substrate perfused.

Panel A- Pyruvate and Lactate- Pyruvate livers had significantly higher ($P < 0.005$) biliary sodium secretion rates as compared to the lactate livers.
Panel B- Pyruvate, Dextrose, and No Substrate- Pyruvate livers had significantly higher ($P < 0.005$) biliary sodium secretion rates than dextrose and no substrate livers. Note: the rate of biliary sodium secretion for dextrose and lactate livers were found not to be significantly different from each other.

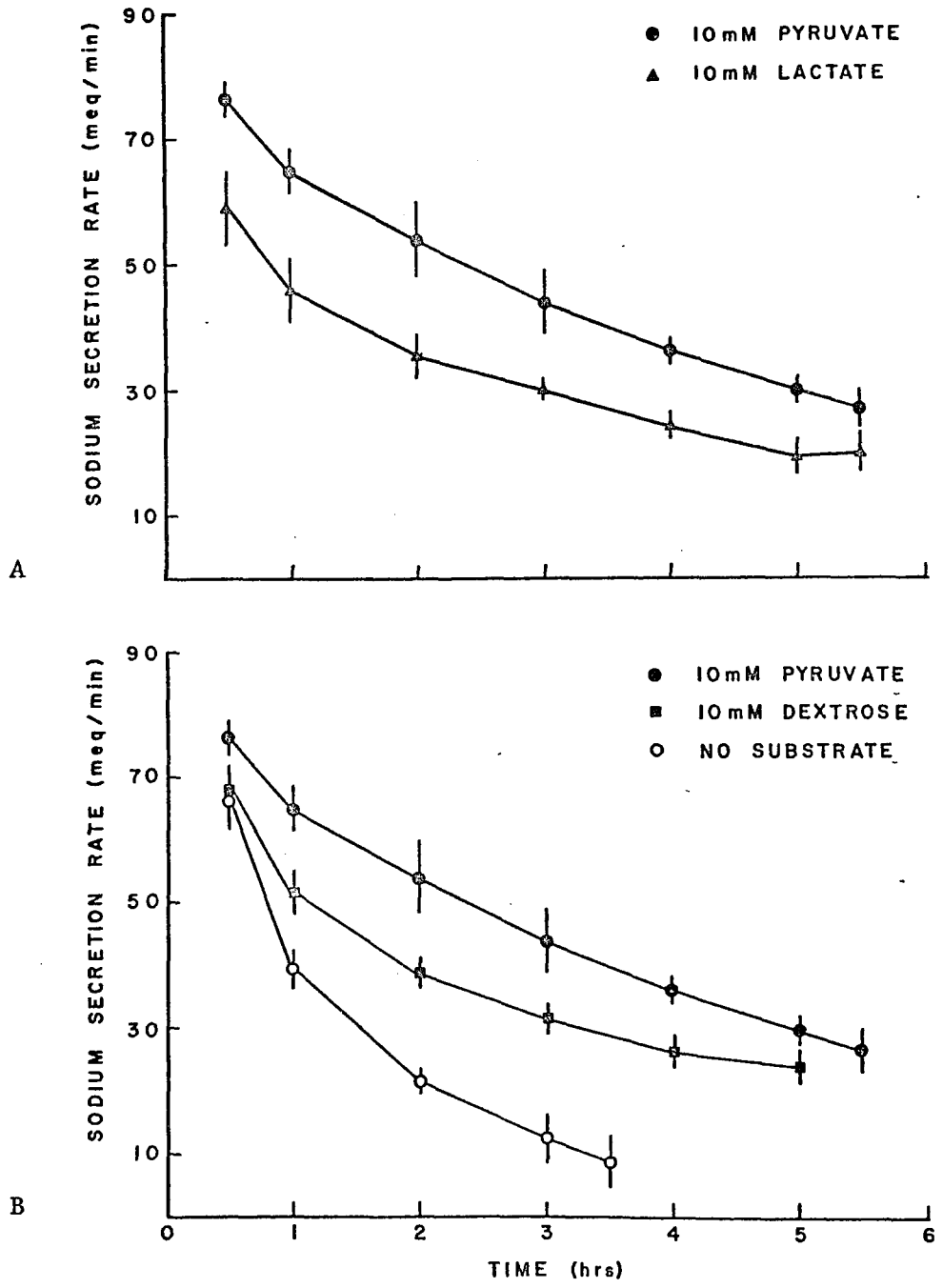


Figure 29. Biliary sodium secretion rates of the isolated perfused rat livers as effected by the particular substrate perfused.

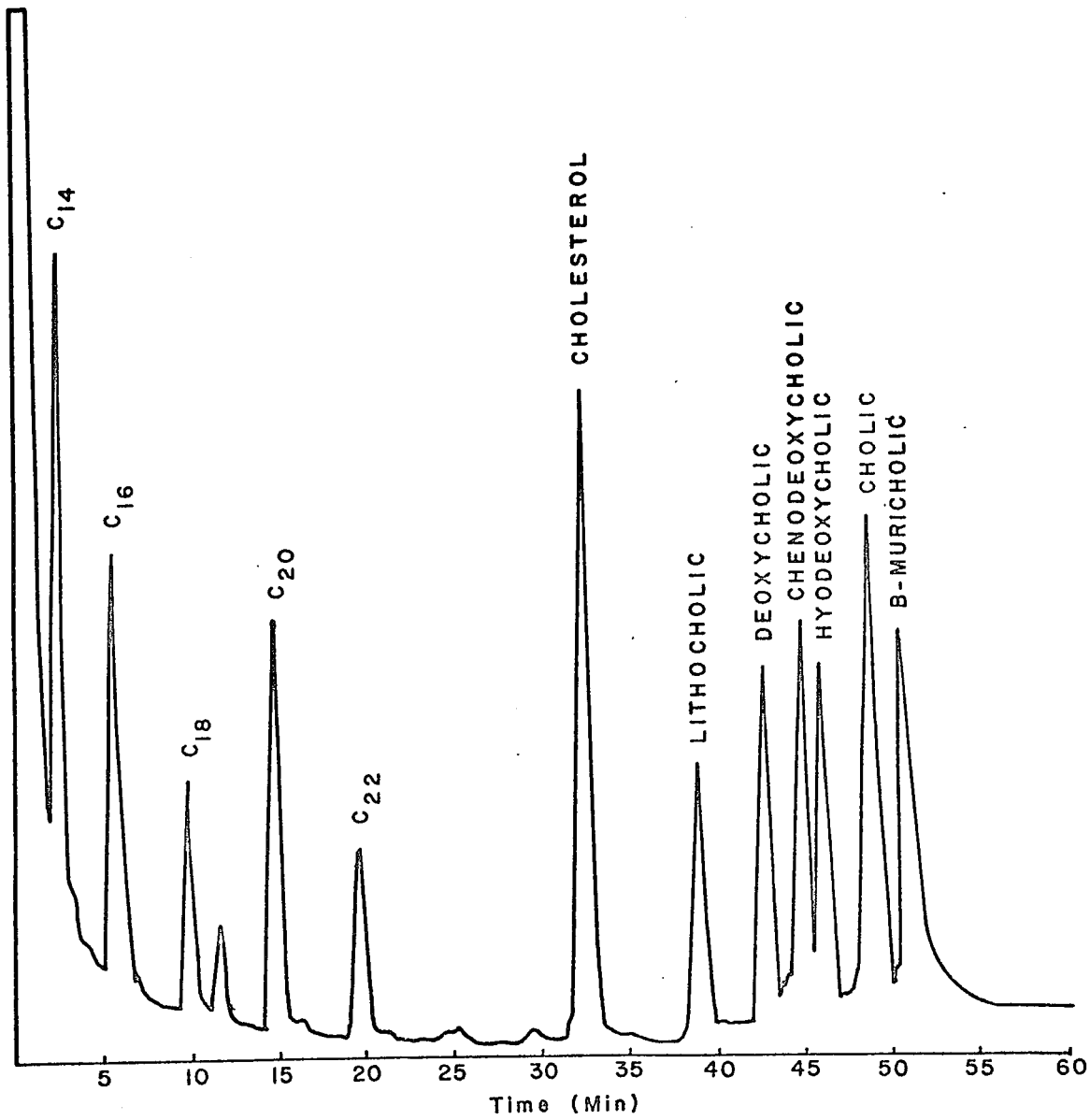


Figure 30. Gas chromatographic separation of a standard mixture of even chained fatty acids, cholesterol and a complex mixture of bile acids.

The method of sample preparation and GLC conditions used for separation are described in the Analytical Procedures of the Materials and Methods.

a standard mixture of individual free fatty acids (even C-14 through C-22), cholesterol, lithocholic acid (3α-hydroxycholanic acid), deoxycholic acid (3α,12α-dihydroxycholanic acid), chenodeoxycholic acid (3α,7α-dihydroxycholanic acid), hyodeoxycholic acid (3α,6α-dihydroxycholanic acid), cholic acid (3α,7α,12α-trihydroxycholanic acid), and β-muricholic acid (3α,6β,7β-trihydroxycholanic acid). Prior to separation by GLC, samples of bile (50 µl) from each perfusion experiment were hydrolyzed, extracted, and derivatized as described in the Materials and Methods section. Comparisons were then made between the substrates perfused and the individual bile acid profiles. Figure 31 shows three typical chromatograms of 50 µl bile samples from livers perfused with pyruvate, dextrose, or lactate at the times indicated. At first the peaks observed in the samples were compared to known standards by computing absolute or relative retention times. Initially all peaks were not positively identified, this was particularly true for the peaks labeled α-muricholic acid, 7-keto-3α,6β-dihydroxycholanic acid, and β-muricholic acid. To be more certain of the identity of these gas chromatographic peaks, gas chromatography-mass spectrometry (GC-MS) was performed on typical bile samples. Except for the case of α-muricholic acid and the 7-keto compound, standard bile acids were obtained and their mass spectrograms compared to those of the peaks that were observed in the bile samples. Originally the bile acids from the bile samples were considered to be only dihydroxy and trihydroxy bile acids . It was thought that the identification of the peaks as dihydroxy and trihydroxy bile acids could be accomplished simply by noteing

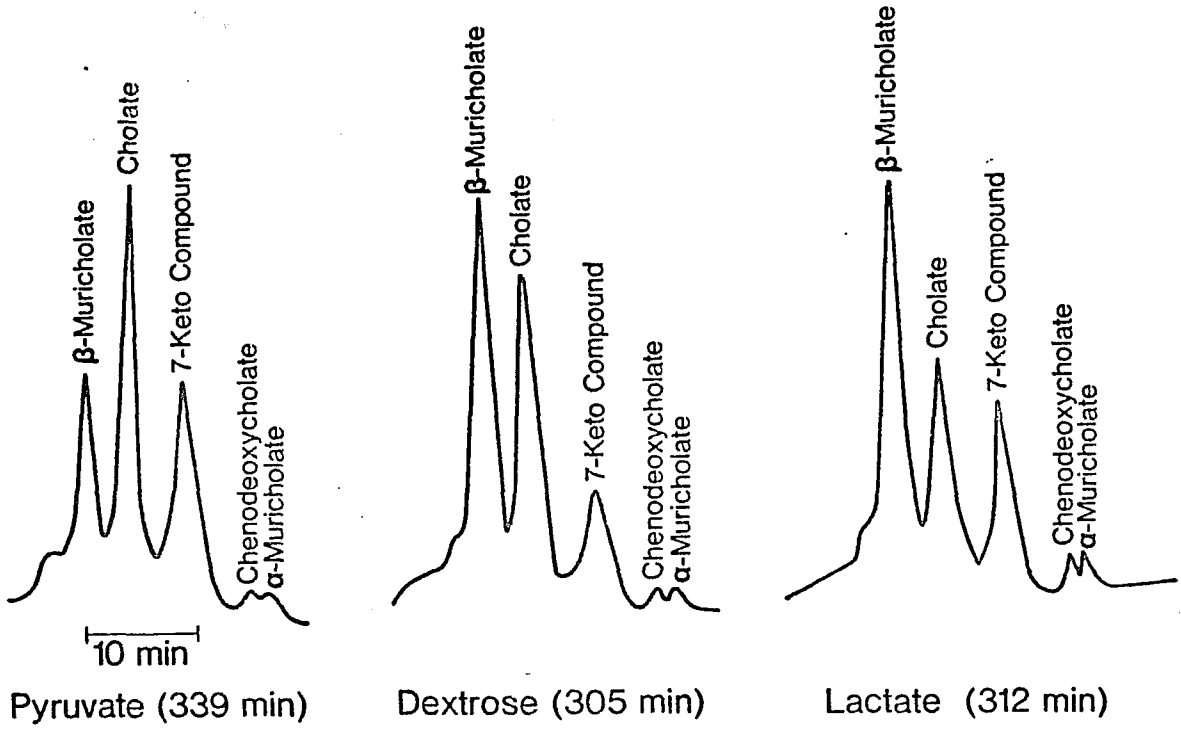


Figure 31. Gas chromatograms of typical bile sample from livers perfused with pyruvate (10 mM), dextrose (10 mM), or lactate (10 mM) at the respective times as indicated.

the molecular ion (M^+) obtained for the respective sample peaks. Of the five GLC peaks (Figure 32) observed during GC-MS (conditions of separation for Finnigan MS procedure) analysis of a typical bile sample, the molecular ion of 710 was found in peaks #1, #4, and #5 identifying these peaks as trihydroxycholanic acids. However, only #2 gave a molecular ion of 596 which indicated that this peak was a dihydroxycholanic acid. Since peak #3 had neither a molecular ion of 710 nor 596, this peak could neither be a dihydroxy nor a trihydroxycholanic acid.

By analyzing the mass spectrograms of five known bile acid standards, lithocholic acid, chenodeoxycholic acid, deoxycholic acid, cholic acid, and β-muricholic acid, particular fragmentation patterns and mass ions were found characteristic of positional arrangements of the trifluoroacetyl groups. When considering the possible combinations of original hydroxyl positions for the bile acid trifluoroacetyl methyl esters, it became evident that the molecular ion minus 49 was a prevalent peak only in cases where there was no fluoroacetyl group (originally a hydroxy group) at position C-12. The 49 mass units were calculated to be the $OCH_3 + H_2O$ splitting off from the molecular ion (Figure 33). It was present in β-muricholic acid ($M^+ - 49 = 661$), chenodeoxycholic acid ($M^+ - 49 = 549$), as well as lithocholic acid ($M^+ - 49 = 437$), but absent in the derivitized deoxycholic acid and cholic acid standards which have in common a trifluoroacetyl group in C-12. This characteristic fragmentation pattern later served in identification of bile acids devoid of a trifluoroacetyl group at C-12 (Figure 33).

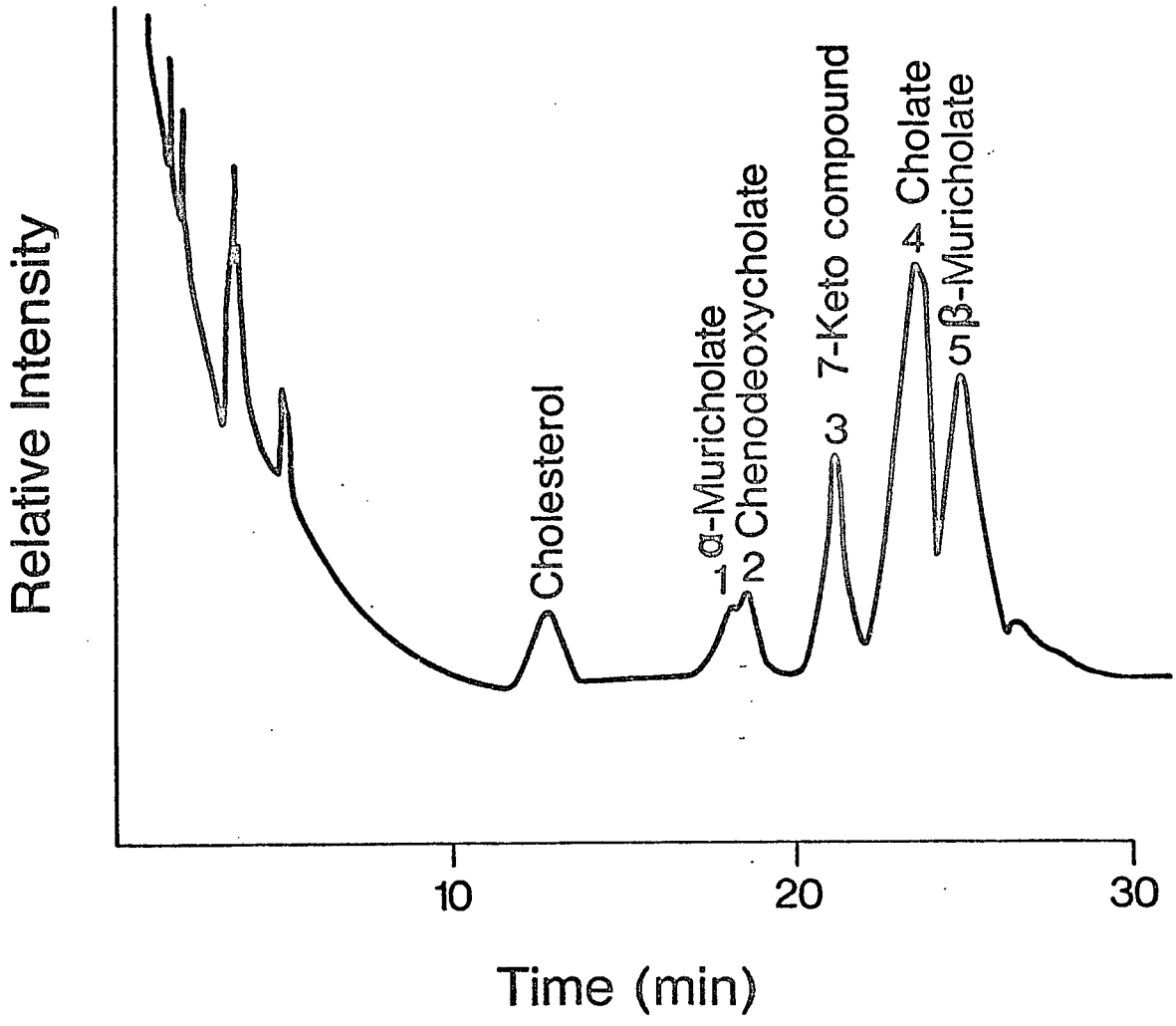


Figure 32. Representative GLC peaks from a typical bile sample as identified by GC-MS.

Samples were run isothermally at 220° on a Finnigan 3300-6100 GC-MS as described in the Analytical Procedures of the Materials and Methods.

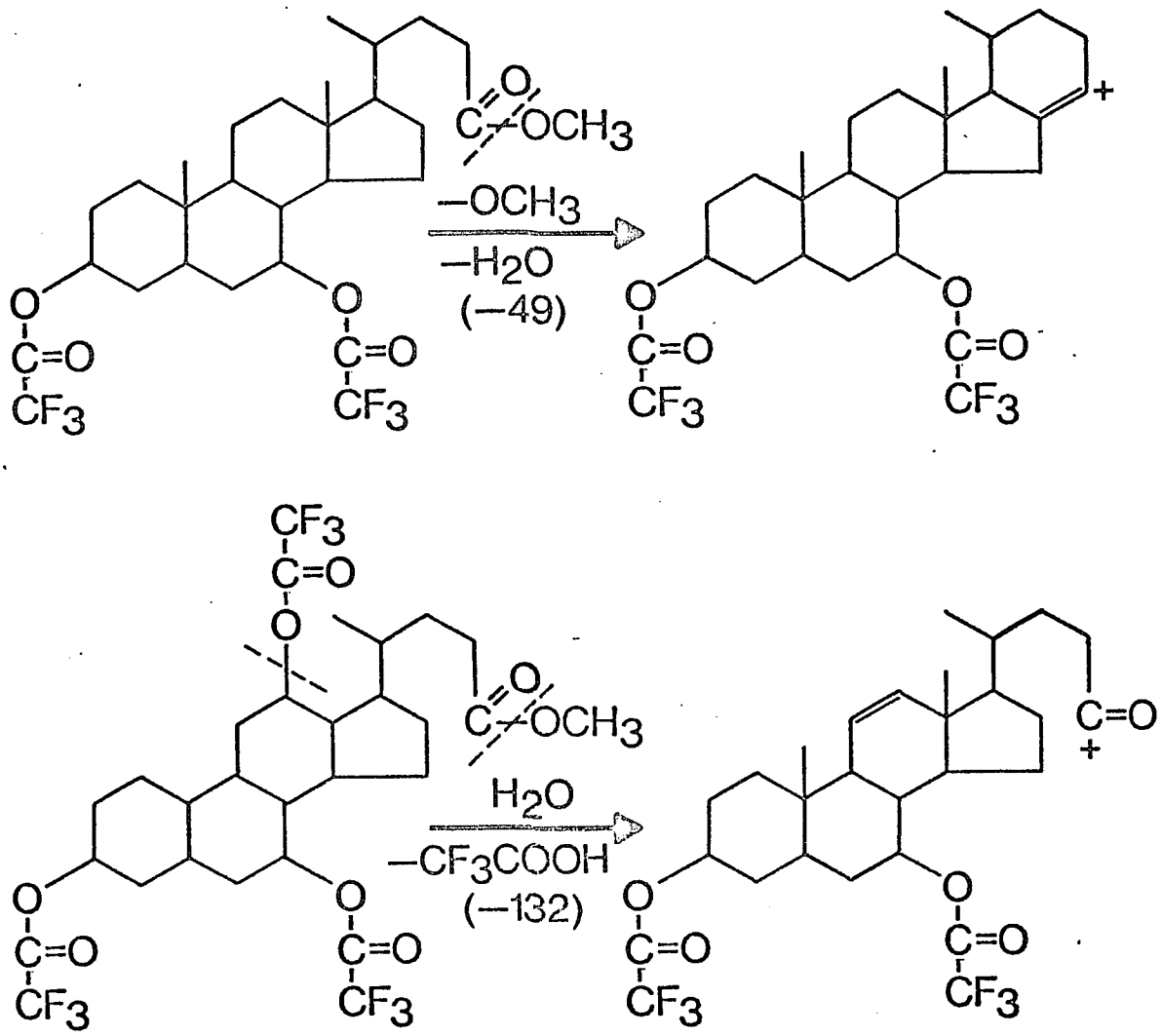


Figure 33. Differences in MS fragmentation of bile acids with and without 12-substitution.

Sample peak #1 (Figure 32) had a prevalent mass ion at $M^+ -$ 49= 661 suggesting that the peak, although representing a trihydroxycholanic acid, did not have a fluoroacetyl group at position C-12. The β-muricholic acid standard was similarly found to have the 661 peak. Sample peak #1 and β-muricholic acid also had in common a fragment at $M^+ -$ 210= 500. This ion arises from a splitting of the third ring (C) of the steroid nucleus between carbons 8,14 and 11,12 and indicated that none of the three fluoroacetyl groups were located in ring C or D, but were located in ring A and B. With use of β-muricholic acid standard, sample peak #5 was identified as β-muricholic acid by GLC retention time as well as by the identity of its MS fragmentation pattern. The GC-MS data demonstrated that peak #1 was a trihydroxycholanic acid with all three hydroxy groups in ring A and B exactly in the same manner as for the βmuricholic acid, but different in stereochemistry from β-muricholic acid as indicated by their separation by GLC. Although no α-muricholic acid could be obtained for comparison purposes, it was concluded from these data and from the fact that α-muricholic has been published to have nearly the same relative retention time (0.85) as chenodeoxycholic acid (0.87) on the liquid phase (QF-1) which was used in these experiments, that in fact peak #1 was α-muricholic acid. Peak #2 was positively identified as chenodeoxycholic acid by the molecular ion at 596 plus the fact that the $M^+ -$ 49= 547 which is characteristic for fragmentation pattern in absence of a 12 fluoroacetyl group. In addition the fragmentation fingerprint was positively identified when compared with that of authentic chenodeoxycholic acid. As stated

above, peak #3 was found to be neither a dihydroxy nor a trihydroxy bile acid. The mass spectrogram of this sample peak showed two characteristic ions of mass 579 and 514. In the transformation of α-muricholic acid to β-muricholic acid a keto intermediate was suspected and would have a molecular ion of 612 if it were 7-keto-3α,6β-dihydroxycholanic acid (Figure 34). Although no M^+ of 612 could be detected, characteristic peaks at mass 579 and 514 were observed. These fragments are postulated to have been formed as indicated in Figure 35. It should be noted that the splitting of water from ketonic bile acid derivatives has been reported to be quite pronounced (Sjovall, Eneroth, and Ryhage, 1971) and may account for molecular instability and the inability to detect the molecular ion. On the basis of this evidence peak #3 was then tentatively identified as the 7-keto, 3α,6β-dihydroxycholanic acid. Peak #4 and #5 were positively identified as cholic and β-muricholic acid respectively by GC-MS comparison with standards. Although both of these peaks represented compounds having the same molecular weight and same number of hydroxyl groups present, the characteristic mass peaks 661 and 523 were used to differentiate them from each other. Both of these mass ions were prominent for the β-muricholic acid but just noticible for the cholic acid peak. This carryover is due to the incomplete separation of these two peaks when run isothermally for GC-MS purposes. The mass ion 661 is $M^+ - OCH_3 - H_2O$ and the mass ion 523 is $M^+ - CF_3COOH$ – sidechain. As has been pointed out by Sjovall (1969) and as indicated by these GC-MS studies, the presence or absence of the 12-hydroxy function produced characteristic

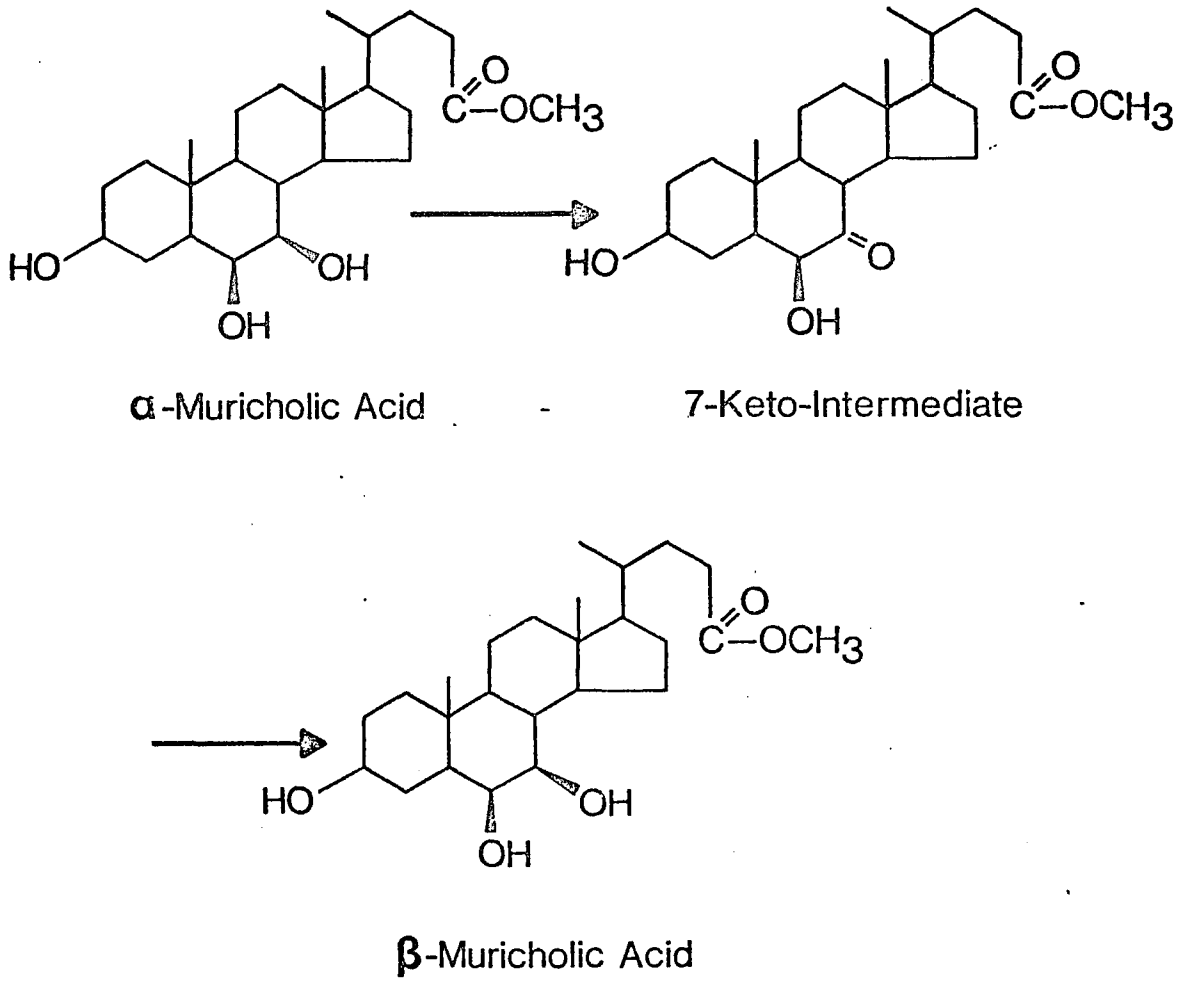


Figure 34. Biotransformation of α-muricholic acid via a 7-keto intermediate to β-muricholic acid.

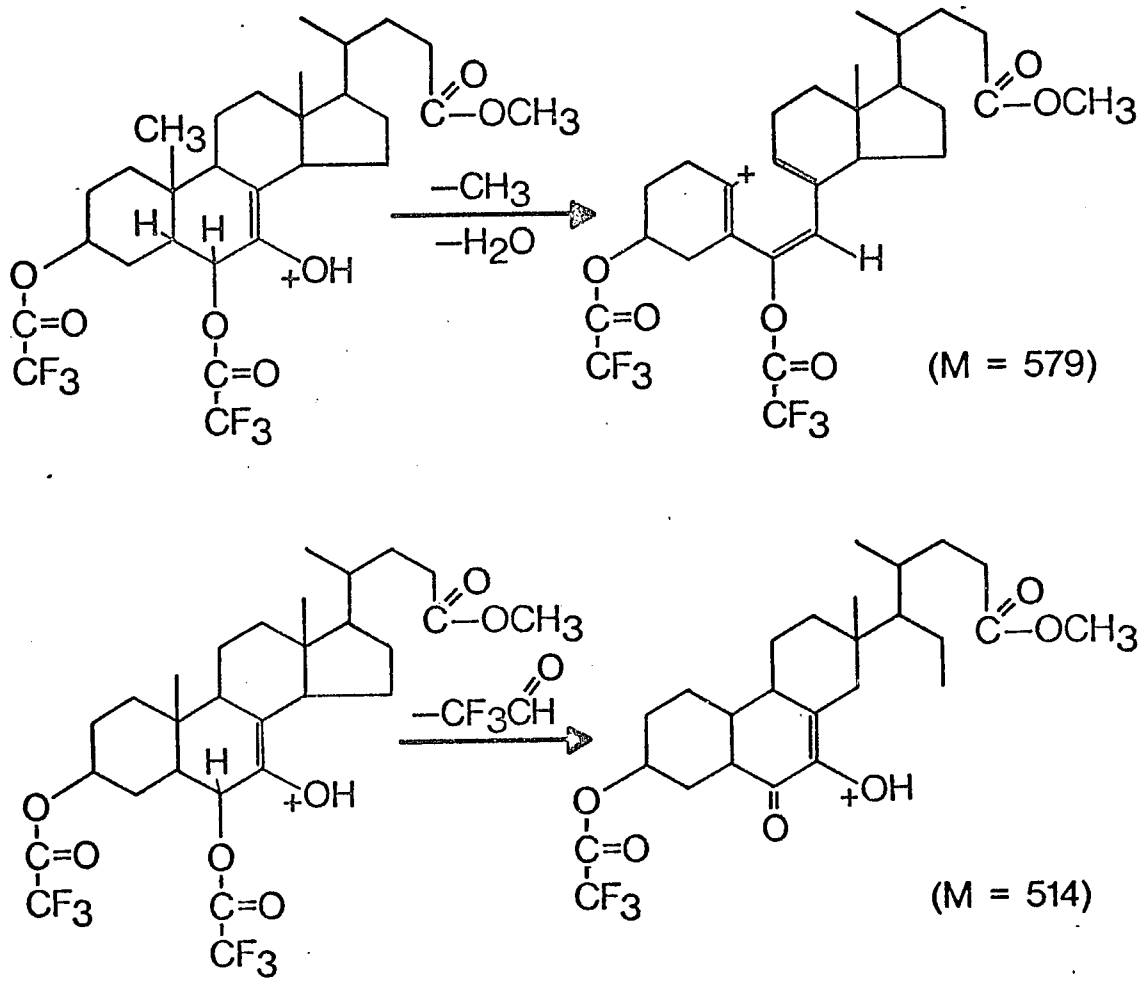


Figure 35. Rearrangements of the tautomer ions of 7-keto, 3α, 6β-dihydroxycholanic acid.

ion fragmentation which can be used for differentiation. In this manner of interpretation the individual bile acids from sample bile were identified.

To return to the considerations of the effect of substrates on bile acid pattern in bile from livers perfused in the presence of lactate, pyruvate or dextrose, it could be shown that the three substrates definitely changed this pattern (Figure 31). The important implication of this finding is that the synthetic pathways for the bile acids might be altered by endogenous changes in substrates available and utilized. It should be mentioned at this point that the rat has a more complex hepatic synthesis of bile acids than the human. Since the rat liver has a very active 6β-hydroxylase (Voight, Thomas, and Hsia, 1968; Voight, Fernandez, and Hsia, 1970), chenodeoxycholic acid is not the synthetic end product of that pathway, as is the case in the human liver, but is further hydroxylated by this enzyme to form α-muricholic acid. The α-muricholic acid is then rapidly transformed in the rat liver to β-muricholic acid through a 7-keto intermediate (Figure 34). Thus the α-muricholic acid, the β-muricholic acid, and the 7-keto-3α, 6β-dihydroxycholanic acid can be considered to be metabolites or end products of the chenodeoxycholate pathway. When comparing the ratio of cholic acid to chenodeoxycholic acid plus its metabolites as affected by the three substrates, it was evident that each substrate produced a different ratio (Table 2). Lactate livers had the lowest ratio, dextrose an increased ratio and pyruvate the highest ratio indicating that lactate is most effective in shunting the synthesis of bile acids

Table 2. Influence of substrate perfused upon individual bile acid synthesis and secretion by the isolated perfused rat liver.

Particular Substrate Used During Perfusion	Average Mean Ratio Chenodeoxycholic Acid Plus Metabolites to Cholic Acid
Lactate (10 mM)	0.310±0.06
Pyruvate (10 mM)	0.845±0.05
Dextrose (10 mM)	0.635±0.034

Bile samples were taken during the 4 to 5 hour perfusion period and analyzed by GLC. Each average mean ratio ± S.E. represents 5 liver perfusions.

toward the chenodeoxycholic acid pathway. On the other hand, pyruvate was the most effective in directing bile acid synthesis toward the cholic acid pathway.

Since the pyruvate livers and to a lesser extent the dextrose livers produced a high oxidative state, which might be considered to be similar to that produced by ingesting large quantities of refined carbohydrates, it became obvious to consider how eventual changes in bile acid ratios in a similar manner in the intact animal could effect cholelithiasis or cholesterol gallstone formation. In more directed terms, if applicable to the intact animal, one could conclude that the higher the oxidative state the more cholic acid is synthesized compared to chenodeoxycholic acid and its metabolites. Since chenodeoxycholic acid has been show to be more effective in maintaining a solubilized bile (Mufson et al., 1974; Redinger and Small, 1972) as well as solubilize existing cholesterol gallstones (Danzinger et al., 1972; Pedersen, Arnfred, and Thaysen, 1974; Hofmann and Thistle, 1974) than cholic acid, it stands to reason that a change in bile acid ratio in favor of cholic acid would produce a potentially more lithogenic bile.

When considering the process by which these substrates could effect such a change in bile acid synthetic patterns, it seemed logical to postulate that the molecular events leading to these alterations are intimately related to changes in cellular redox potentials. As has been previously indicated these differences in redox potential might be really extreme in this system by using the dialysis unit in which a constant supply of substrate will not allow a natural redox state or

equilibrium to occur, but will drive the redox state far in the direction of its biochemical limit. In physiological states redox potentials never vary to the extremes produced in these perfused livers, however, even slight changes may be significantly high enough over long periods of time to influence bile acid patterns even under these conditions. In addition, Shefer et al.(1973) demonstrated that the administration of taurocholic acid and taurochenodeoxycholic acid at a level of 1% of the diet had quite different effects. Taurocholate depressed the cholesterol-7α-hydroxylase enzyme activity by 70%, but taurochenodeoxycholate did not. Recent evidence from clinical studies in man have also demonstrated that cholate and to a greater extent its intestinal metabolite, deoxycholic acid, selectively inhibits chenodeoxycholic acid synthesis (Pomare and Low-Beer, 1975). This supports the existance of a selective feedback mechanism on the catabolic processes of cholesterol metabolism to bile acids. This means that in addition to the initial changes in ratios of bile acids (which will affect cholesterol solubilization), there is a secondary effect in parallel and in the same direction such that increased cholate leads not only to decreased cholesterol conversion and increased cholesterol levels, but further promoting the maintenance of the altered bile acid ratio. These factors, as they change the bile acid ratio in favor of cholic acid, augment the chance of forming lithogenic bile and thus potentiating gallstone formation.

Information has indicated that many Americans, possibly by virtue of high carbohydrate diets, are normally very near the limit of

the ABC saturation line depicted previously in the phase diagram (Figure 1). It has also been stated by these authors (Grundy, Ahrens, and Salen, 1970) that the ABC saturation line may regularly be exceeded during fasting and over short periods during the sleep cycle for normal individuals without any apparent radio-opaque gallstones. It is not difficult to believe that any factor that would cause an increase in cholic acid would subsequently precipitate all the events described thus increasing the likelihood of periods of super-saturation with the danger of initial precipitation, nucleation, or sustained growth of preexisting cholesterol gallstones. Changes in favor of a decreased chenodeoxycholic acid pool size have been documented for gallstone patients (Heller and Boucher, 1973; Bennion et al., 1976). In addition gallstone patients have been found to have decreased cholesterol conversion to bile acids (Heller and Boucher, 1973; Pedersen, Arnfred, and Thaysen, 1975) and reduced circulating total bile acid pool size (Pomare and Low-Beer, 1975).

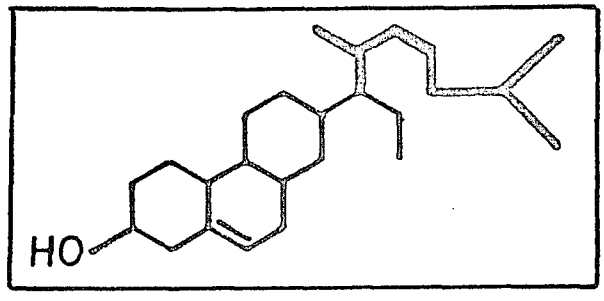
Since it has been postulated that redox state is instrumental in the changes observed in the bile acid patterns the obvious questions are then, how can substrates of different oxidative potential regulate or influence the bile acid pathways, and where would the site of action be located?

In order to study the effects of substrates upon the overall process of cholesterol turnover in a more direct and more physiological fashion, including experimentation, a [24,25-^{3}H]-cholesterol was synthesized from unlabeled desmosterol as previously described in the Materials and Methods section. Since all bile acids (24 carbons) are

the result of the loss of the terminal three carbon units from the sidechain of the cholesterol molecule (27 carbons), the rate of cholesterol sidechain oxidation can be considered identical to the rate of cholesterol turnover (Mitropoulos and Myant, 1965). Figure 36 illustrates the locus of tritium in the sidechain of the parent compound.

The advantage of this labeled cholesterol is that upon cholesterol sidechain oxidation (CSO) the respective hydroxylases and dehydrogenases release tritium from the compound into $^{3}H_2O$ via NADH (NADPH) and/or FADH. Several pathways (Cronholm and Johansson, 1970) have been proposed for the sequence of reactions occurring during sidechain oxidation. Figure 37 demonstrates that for each proposed pathway the route of degradation, whether by mitochondrial or microsomal processes, results in the liberation of ^{3}H to NAD (NADP) or FAD which is ultimately transferred to $^{3}H_2O$. The tritiated water produced is freely exchangeable with cellular and extracellular water. Thus the rate of tritiated water appearance in the perfusion medium of an isolated liver is identical to the rate of cholesterol sidechain oxidation and therefore the cholesterol turnover rate in the isolated liver. This particular method of measurement is thus independent of the additional processes of conjugation, cellular compartmentalization, nonspecific protein binding, carrier protein binding, and secretion. Using this labeled compound, it was possible to study the rate of cholesterol turnover under the influence of different substrates (redox states) in the isolated rat liver system.

[24,25 ^{3}H] Cholesterol



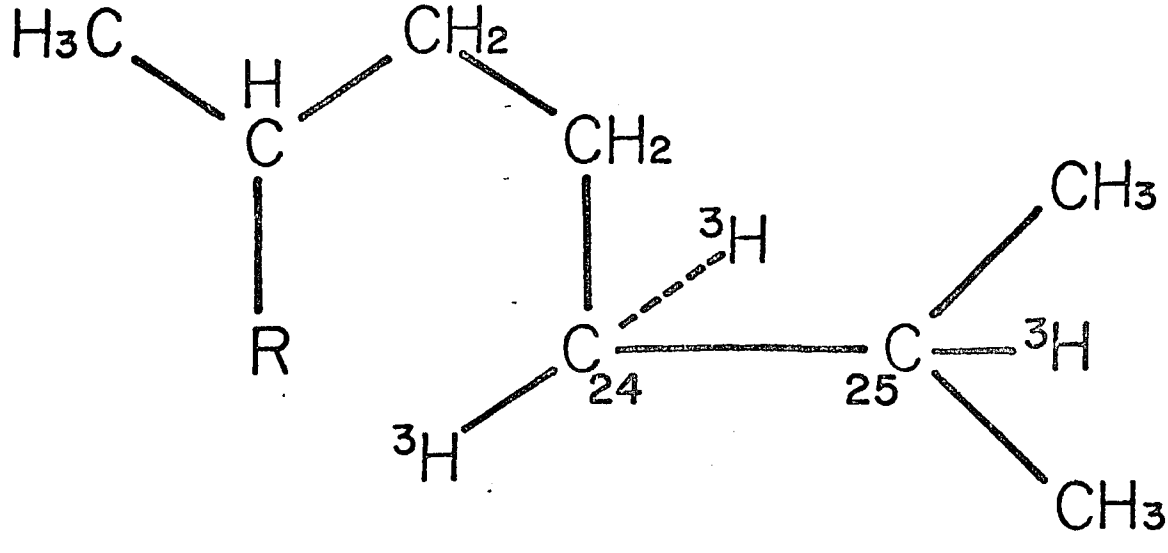


Figure 36. Illustrated [24,25-^{3}H]-cholesterol - positional location of tritium in the sidechain.

Note: tritium was introduced by reduction of C-24, C-25 double bond therefore only one tritium would be present at C-24 and one at C-25. At C-24 the tritium can be in either the alpha or beta position.

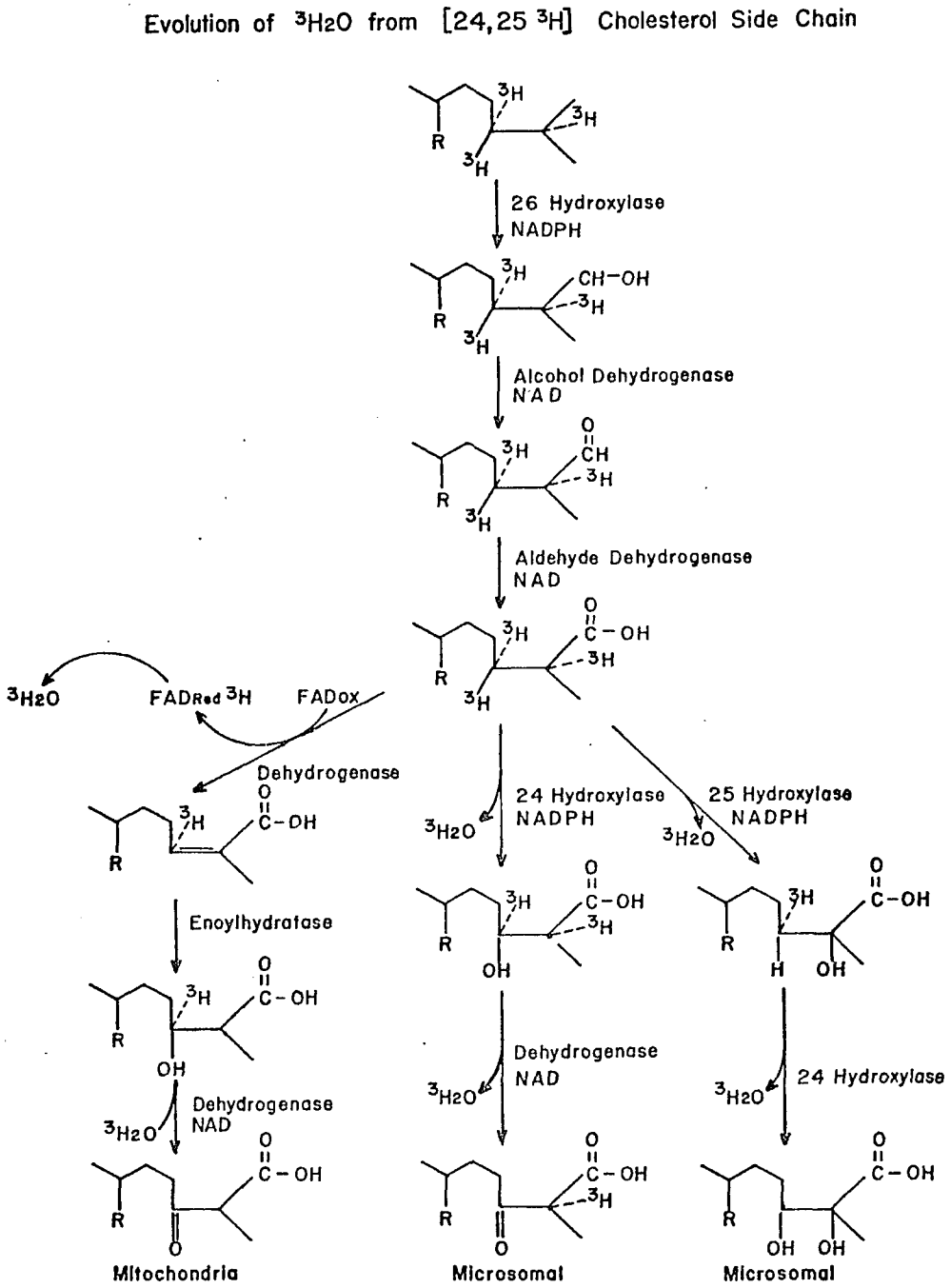


Figure 37. Evolution of ^3H_2O from [24,25-^3H]-cholesterol sidechain by way of proposed mitochondrial or microsomal pathways.

The first perfusion was done solely to demonstrate the rate of [24,25-^{3}H]-cholesterol uptake by the isolated liver (Figure 38). Ninety-eight percent of the administered radioactivity was taken up rapidly by the liver over a 60 minute period in an exponential fashion. In subsequent liver perfusions, one ml of perfusion medium was collected at 15 to 30 minute intervals and run through a mixed bed Dowex resin column (as described in the Materials and Methods section) and the water eluted from the column counted for tritium.

To quantitatively measure the tritium water produced by oxidation of the labeled cholesterol the dialysis unit was disconnected so that the tritium water produced would not be dialyzed out of the circulating buffer circuit. Experiments were performed in livers which had been pre-labeled with radioactive cholesterol and then perfused alternately during consecutive 60 minute recirculatory periods with lactate or pyruvate. The perfusion periods were interspaced with 10 minute washout periods in a non-circulatory mode. In each washout period the liver was rinsed with 500 ml of substrate and buffer used in the following perfusion period (Figure 39). As indicated pyruvate perfusion periods elicited a higher rate of cholesterol sidechain oxidation demonstrating that within the cell the process of cholesterol turnover was increased in response to the higher oxidative state. This finding was somewhat unexpected, especially when considering the fact that the long term perfusion studies showed no difference in total bile acid synthesis and secretion. There are several possible explanations for this discrepancy. One, the rate of cholesterol turnover to bile

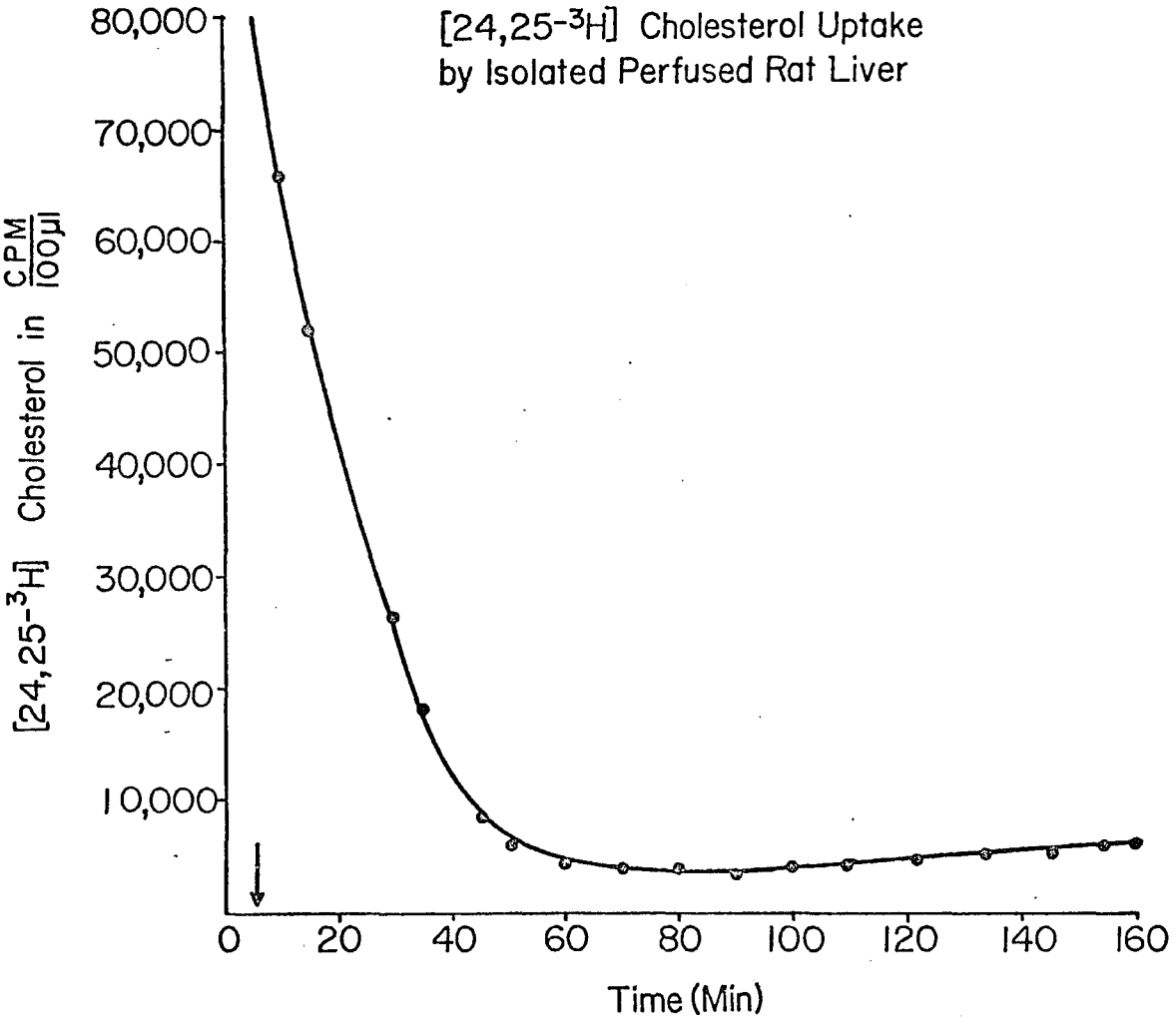
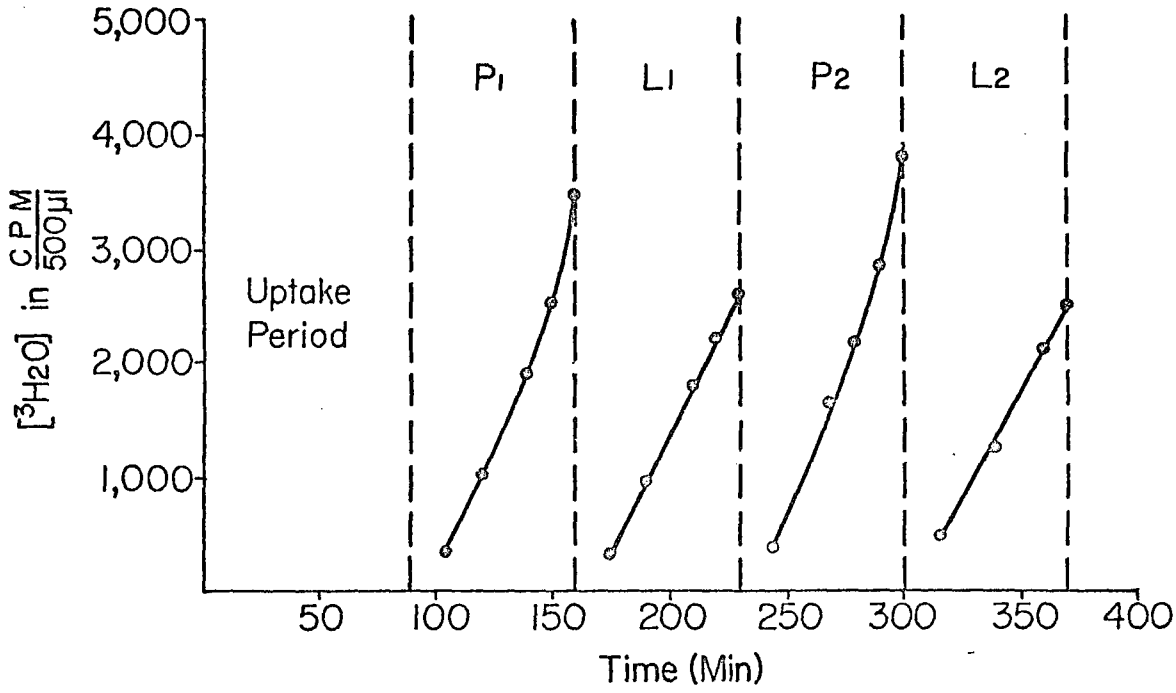


Figure 38. Uptake of [24,25-^{3}H]-cholesterol by the isolated perfused rat liver.

At the arrow 0.155 µCi of [24,25-^{3}H]-cholesterol was introduced into the circulating buffer.



Buffer	(N)	Mean % Decrease ± S.E.
P1→L1	5	19.96±1.3
P2→L2	5	25.60±2.9

Figure 39. Rate of $^{3}H_2O$ production as influenced by alternating pyruvate and lactate perfusion buffers in isolated perfused rat livers.

P-1 to L-1 and P-2 to L-2 represent the differences in $^{3}H_2O$ production when comparing the respective perfusion periods as influenced by changing substrate from pyruvate to lactate.

acids, while being affected by changes in oxidation rate, is not the rate limiting factor in the secretion rate of bile acids into the bile and the possibility exists that a decreased conjugation of the newly synthesized bile acids affects secretion rates. Although free bile acids are excreted into bile (usually less than 5%), O'Maille, Richards, and Short (1967) have shown that the conjugation process can be limiting in bile acid secretion, therefore if taurine and glycine levels are depleted in this system the conjugation process could be the limiting factor. Two, although more bile acids are formed, cellular compartmentalization or binding is limiting in respect to secretion rates. Normally the liver maintains a pool of bile acids (Wheeler, Mancusi-Ungaro, and Whitlock, 1960) which is much larger, due to the enterohepatic circulation, than in the case of the isolated liver where only newly synthesized bile acids represent this pool. Liver has also been shown to have cellular binding proteins (Levi, Gatmaitan, and Arias, 1969) for the bile acids. If the level of bile acids present within the liver is below a certain point the newly synthesized bile acids are not directly secreted but are first distributed and bound in the cellular pool. The rate of secretion would then be a function of the equilibrium set up between nonspecific cellular binding and release. Thus differences in the quantity of total bile acids synthesized (at this low level) would be dampened by the non-specific cellular binding. Three, the particular route (mitochondrial or microsomal) of cholesterol sidechain oxidation was influenced. The original concept was that only one of the postulated pathways was functional. But

if both are active pathways it is possible that potentially more tritium be released from the labeled compound when channeled through the mitochondrial pathway as opposed to the microsomal pathway. As illustrated in Figure 37 the microsomal 24-hydroxylase pathway would have a 50% chance of losing both tritium molecules, whereas the other microsomal pathway and the mitochondrial pathway would lose both tritium molecules. This possibility would only hold true if the three carbon unit cleaved is not further metabolized. Since propionate has been proposed to be the resultant cleaveage product (Mitropoulos, Myant, and Gompertz, 1970) and since the isolated perfused liver has been shown to efficiently oxidize propionate (Gonzales de Galdeano et al., 1973), it is not necessary to consider this possiblility as very feasible. Although additional work is needed to delineate the complex mechanisms involved in this discrepancy, the fact remains that the use of the [24,25-^{3}H]-cholesterol in conjunction with perfused livers, as well as other experimental systems, will further allow the study of other endogenous and also exogenous agents which might act directly upon cholesterol sidechain oxidation and thus cholesterol turnover. The results from such experiments will also provide a more complete characterization of cholesterol metabolism to bile acids.

To test the consistancy of these findings, the use of the labeled cholesterol was extended to isolated suspended liver cells. Isolated liver cells were obtained by the method of Hayes and Brendel (1976). To each vial of suspended liver cells 1.8 μCi of labeled cholesterol was added. Lactate or pyruvate concentrations were added at

zero incubation time to make the final concentration 10 mM. The uptake of [24,25-^{3}H]-cholesterol by the cells and appearance of tritiated water over an 8 hour incubation period as effected by lactate and pyruvate are shown in Figure 40. As was found in the isolated perfused livers, pyruvate was observed to produce a significantly higher CSO rate than lactate in the isolated cell preparations, again supporting the concept that the higher the oxidative potential the higher the CSO rate and cholesterol turnover.

Since thyroid hormones are known to effect the rate of bile acids synthesized (Strand, 1963; Uchida et al., 1970), D- and L- thyroxine added to isolated liver cells were used to test the adaptability and usefullness of this tool. Figure 41 shows the effects of D- and L-thyroxine upon the evolution of $^{3}H_2O$ from radioactive cholesterol when dextrose (5 mM) was the substrate. Although there seems to be a higner CSO rate with the L-thyroxine ($P < 0.05$) treated cells, no significant difference was found between control cells and D-thyroxine treated cells. The lack of effect with the D-thyroxine could possible have been due to the dosage used. Another reason for a reduced response in both D- and L-thyroxine treatment could have been the long onset of action needed for these compounds. In order to allow for a longer onset of action period, intact rats were pretreated with either 100 mg/kg L-thyroxine or 400 mg/kg D-thyroxine for 5 days. Thyroidectomized and control animals were administered 0.9% saline injections in the same sequence as the thyroid treated animals. On the 6th day 0.56 µCi

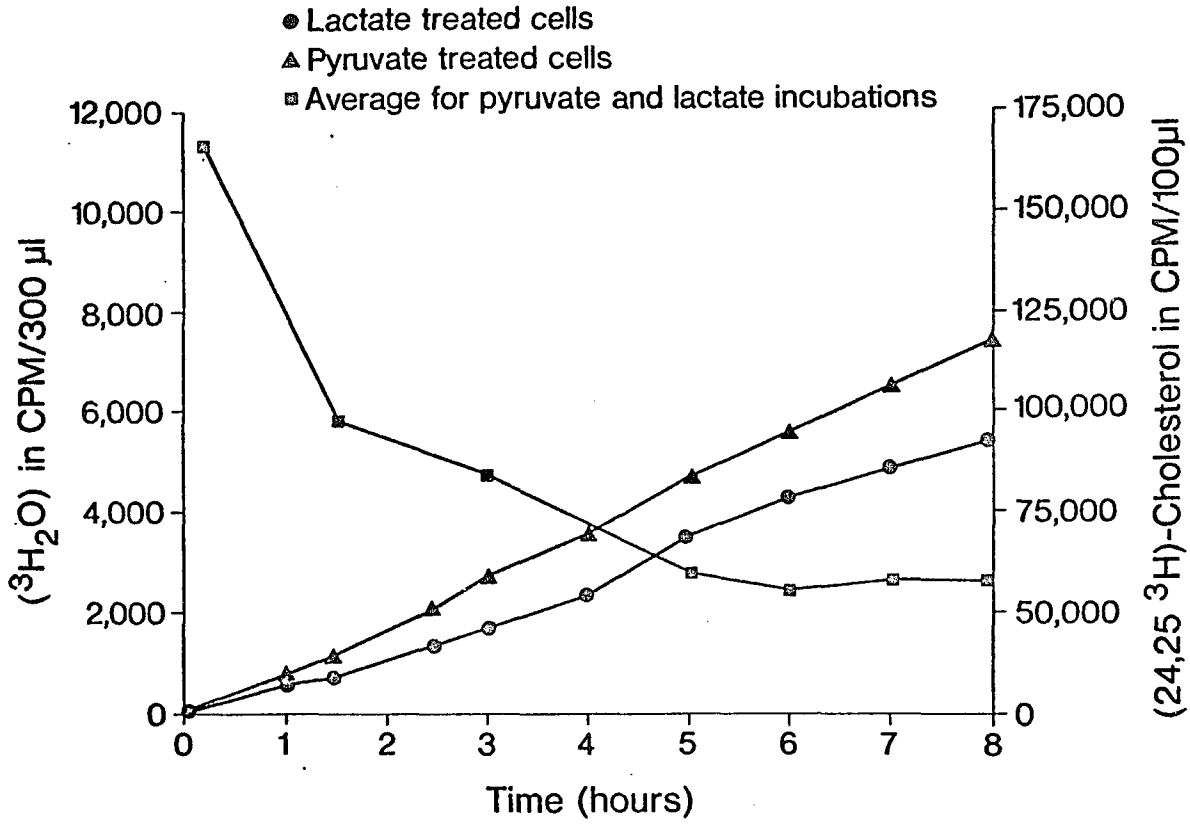


Figure 40. Rate of [24,25-^{3}H]-cholesterol uptake and ^3H_2O appearance as influenced by pyruvate and lactate substrates in isolated suspended hepatocytes.

[24,25-^{3}H]-cholesterol, 1.6 µCi/incubation flask, was introduced at zero incubation time. Each line represents 5 experiments at which all time points beyond 3 hours were significantly different ($P < 0.01$) from each other.

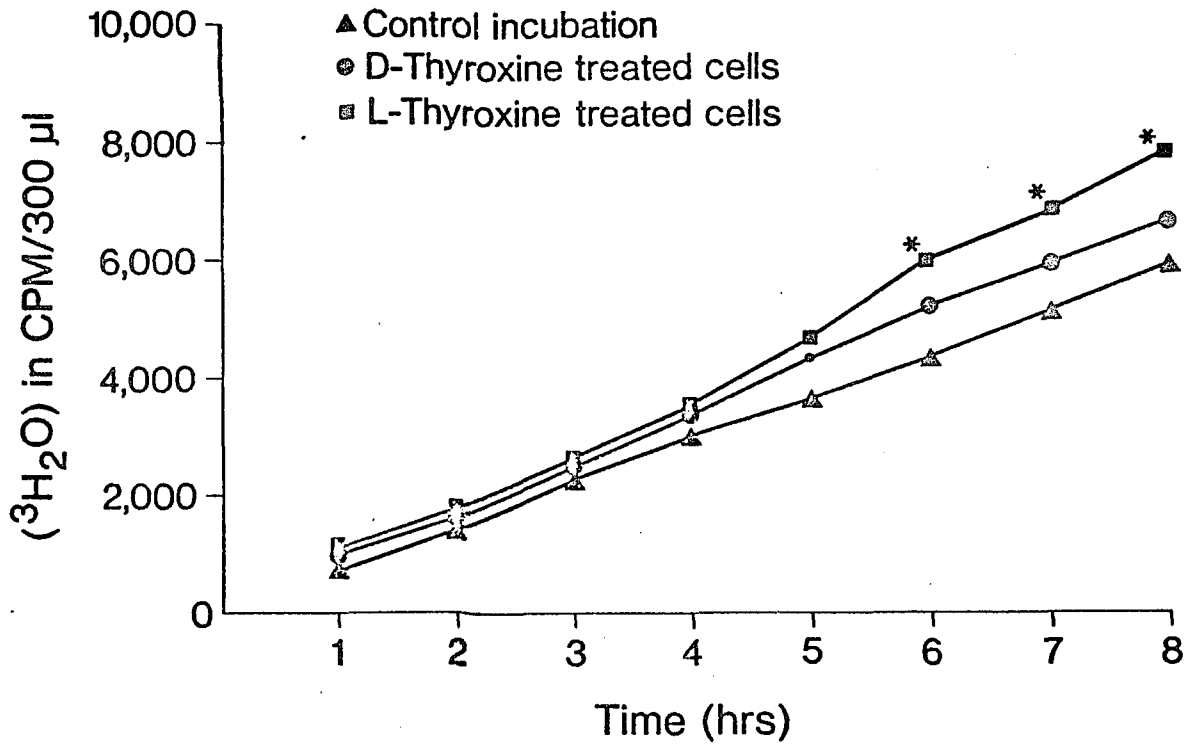


Figure 41. Influence of D- and L-thyroxine upon the rate of ^3H_2O appearance in the incubation medium of isolated suspended hepatocytes.

[24,25-^{3}H]-cholesterol, 1.6 µCi/incubation flask, was introduced at zero incubation time. L-thyroxine, 0.04 µg/ml and D-thyroxine, 0.01 µg/ml were added at time zero. L-thyroxine treated cells produced significantly higher ($P < 0.01$)* tritium water than controls as indicated. Each line represents the mean average for three experiments.

[24,25-^{3}H]-cholesterol/100 g animal weight was administered i.v. via the tail vein. Figure 42 illustrates the differences observed in urinary $^{3}H_2O$ excreted as related to the individual types of treatment. The treatment with drug or saline of the particular animal groups was continued throughout the experimentation period. A clear relationship is seen between the thyroid activity and the amount of CSO observed substantiating the relationship between thyroid activity and rate of cholesterol catabolism to bile acids. The amount of CSO for L-thryoxine treated animals was observed to be 71% higher than control animals over the experimentation period (12 days). For D-thyroxine treated animals it was 27% higher than controls. In contrast, thyroidectomized animals had CSO rates 58% that of the control animals. These results definitely demonstrate the validity of the usefullness of this unique tool in the determination of cholesterol turnover in a variety of preparations.

The thyroid treatment was considered to produce a high oxidative state similar to that found in the livers perfused with pyruvate buffer, thus one would expect to see a similar increased cholic acid synthesis in these thyroid treated animals. However, Strand (1963) found that chenodeoxycholic acid increased preferentially to cholic acid after D-and L-triiodothyroneine treatment. A possible explanation for this contradiction is that the bile acid identification as chenodeoxycholic acid was not only chenodeoxycholic acid but also deoxycholic acid. If in fact the thyroid hormone treatment acted to increase the rate of cholic acid synthesis, as implied from these studies,

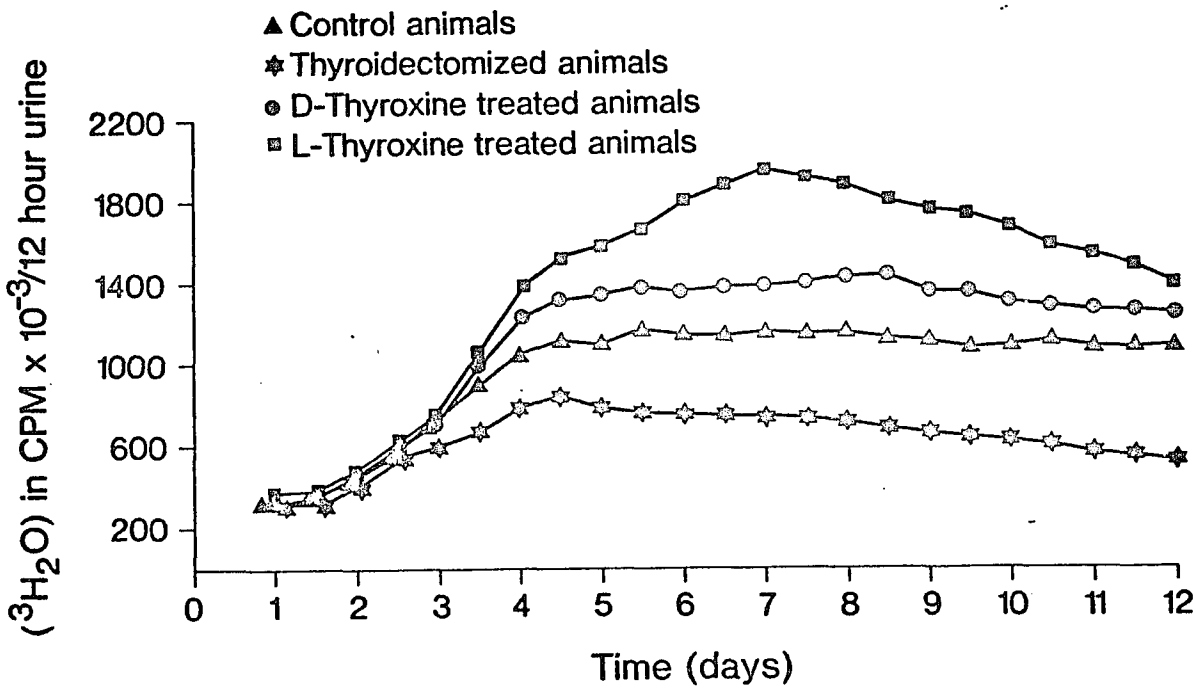


Figure 42. The effect of thyroid state *in vivo* upon the rate of ^3H_2O appearance in the urine of rats.

Following 5 days of respective pre-treatments, [24,25-^{3}H]-cholesterol (0.56 µCi/100 g animal weight) was administered i.v. via the tail vein to each rat. Each point represents the mean average of two rats. Each group of animals subjected to the respective treatments were significantly different ($P < 0.01$) from each other over the experimentation period.

would be a concomitant increase in deoxycholic acid formed from cholic acid during enterohepatic circulation. Since deoxycholic acid and chenodeoxycholic acid are both dihydroxy bile acis and have similar chemical properties these two compounds would appear at the same point on paper chromatography with the method used by Strand (1963) to separate chenodeoxycholic and cholic acid. Further support of this idea is that Hellstrom and Lindsteadt (1964) demonstrated the existance of a direct relationship between the thyroid state and cholic acid turnover. Higher thyroid doses produced faster cholic acid turnover. Realizing that the total cholic acid quantity is increased during thyroid treatment as well as the increased turnover rate suggests that more deoxycholic acid was present and in Strand's case mis-identified as chenodeoxycholic acid. Unfortunately bile samples were not collected from any of the thyroid treated animals studied to clarify this point.

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